ECOLOGY AND DISTRIBUTION OF *ALFALFA MOSAIC VIRUS* (AMV) AND *TRIFOLIUM REPENS* FOR THE ECOLOGICAL RISK ASSESSMENT OF GENETICALLY MODIFIED AMV-RESISTANT *T. REPENS*

by

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DECLARATION

The research presented in this thesis is, except where otherwise acknowledged in the preface, my own original work. Technical assistance, advice and supervision is referred to in the acknowledgements.

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PREFACE

The research outlined in the following five chapters was principally completed by myself, specifically for the purpose of this thesis. All data chapters (chapters two, three and four) are formatted for publication.

Chapter two is based on a manuscript accepted by Ecological Applications in June 2011:

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Contributions: All authors contributed conceptually. I planted and maintained white clover plants in glasshouses, undertook all virus tests, gathered all raw data, undertook some data analysis and was responsible for manuscript completion. Dr. Robert Godfree was involved in fieldwork planning, also provided assistance with fieldwork, the majority of data analysis for the paper and the construction of Table 1, Figure 2.2 (in part), 2.3, 2.4 and 2.6- which were adopted for use in the thesis chapter.

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Contributions: All authors contributed conceptually. Dr. Celeste Linde assisted with experiment and analysis planning. I undertook the preparation of all samples for

sequencing, sequence alignment, sequence editing, sequence analysis and manuscript completion.

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existence and leading the individual towards freedom." It has been a significant challenge but a great pleasure to complete my thesis. I am thankful to all that have assisted in the process of "ennobling" my life through the process of my PhD.

ABSTRACT

This thesis presents a study of the ecology, distribution and pathology of the pasture species *Trifolium repens* and its pathogen *Alfalfa mosaic virus* (AMV) in south-eastern (SE) Australia, to inform an ecological risk assessment of transgenic AMV-resistant *T. repens.* There are concerns worldwide regarding the environmental release of pathogen-resistant (P-R) pasture plants as pasture species have a history of invasiveness. The key concern is that following release from pathogen pressure, P-R plants may become weedy.

The results of this work indicate that *T. repens* is naturalised in many habitats in SE Australia (at 59% of 213 sites visited in a 300,000 km^2 study region). AMV was detected at 15% of sites, was not restricted by region or habitat type, and was significantly more likely to occur in naturalised *T. repens* populations that were abundant, close to agriculture, and/or disturbed. Coat protein (CP) analysis of AMV (83 isolates) from naturalised *T. repens* in SE Australia suggests that the population has little structure by geographic origin, host or community type, suggesting that AMV dispersal is largely human mediated in this region. None of the isolates tested had a CP nucleotide sequence identical to the transgene used for GM *T. repens*, but most (71%) possessed the same amino acid sequence. The remaining isolates had CP sequences that differed by up to four amino acids to the transgene. Transgenic *T. repens,* if grown in SE Australia, is likely to be exposed to the full suite of AMV variants observed. Therefore, prior to environmental release, resistance of transgenic *T. repens* to isolates representing the genetic diversity present in SE Australia should be evaluated. Naturalised and cultivated *T. repens* genotypes were susceptible to AMV infection and AMV generally reduced *T. repens* growth (mean reductions up to 35%). Variability in the infectivity of AMV isolates and host-isolate specificity were

observed; growth impacts varied depending on individual clover x virus combinations, indicating that AMV may be more important in reducing host population size for some clover genotypes than others.

This thesis illustrates the complex nature of ecological risk assessments of widespread invasive pasture species and demonstrates the need for targeted habitat- and pathosystem-specific assessments. Completion of initial stages of this risk assessment suggest that AMV resistance is likely to increase the fitness of naturalised *T. repens* populations, and so AMV-resistant *T. repens* may pose a risk to some native habitats in SE Australia.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

A	Alanine
ACT	Australian Capital Territory
AgDryWt	Mean above ground dry weight (g)
AMOVA	Analysis of molecular variance
AMV	*Alfalfa mosaic virus*
ANOVA	Analysis of variance
ANU	The Australian National University
BranchNo	Mean number of branches on the longest stolon (only branches that resulted in secondary stolons ≥20 mm)
Bt	*Bacillus thuringiensis*
BYMV	*Bean yellow mosaic virus*
C1	Census 1 (one month old plants)
C2	Census 2 (two month old plants)
C3	Census 3 (three month old plants)
ClYVV	*Clover yellow vein virus*
CMV	*Cucumber mosaic virus*
CP	Coat protein
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribonucleic acid
E	Glutamic acid
FET	Fisher's exact test

FHtot	Mean total number of flower heads
G	Likelihood ratio test statistic
G	Glycine (Chapter 3 only)
GM	Genetically modified/ genetic modification
GTR	Gene Technology Regulator
H	Histidine
H-P	Host-pathogen
I	Isoleucine
InfHt	Mean flower head height (mm)
InfWid	Mean flower head width (mm)
Inleng	Mean internode length (mm) measured 50 mm from the end of the longest stolon
Lfleng	Mean leaflet length (mm) measured on the middle leaflet of the third or fourth fully expanded leaf from the end of the longest stolon
Lfwid	Mean leaflet width (mm) measured on the middle leaflet of the third or fourth fully expanded leaf from the end of the longest stolon
Lvs	Mean total number of leaves
Maxht	Mean maximum plant height (mm)
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NJ	Neighbour joining
Nm	Number of mutants
NP	National Park

NSW	New South Wales
OGTR	Office of Gene Technology Regulator
PBS	Phosphate buffered saline
PCA	Principal coordinates analysis
PCR	Polymerase chain reaction
PhiPR	Correlation between sequences within sites, relative to sequences from the same region
PhiPT	Correlation between sequences within sites, relative to the total
PhiRT	Correlation within regions, relative to the total
Pi	Nucleotide diversity
PR	Pathogen-resistant
PriStNo	Mean number of primary stolons ≥20 mm
PRSV	*Papaya ring spot virus*
Q	Glutamine
RNA	Ribonucleic acid
Rootwt	Mean root dry weight (g)
Rsratio	Mean root to shoot ratio (g)
RT-PCR	Reverse transcriptase-polymerase chain reaction
RVE	Relative virus effect, a measure of a growth parameter in response to AMV infection when virus free (V-) and virus infected (V+) clones are compared, calculated as the % RVE = ((V+/V-)-1)*100
SE	South Eastern
StolL	Mean length of the longest stolon (mm)

StThick	Mean internode thickness (mm) measured approximately 50 mm from the end of the longest stolon.
T	Threonine
TEV	*Tobacco etch virus*
TMV	*Tobacco mosaic virus*
ToRMV	*Tomato rugose mosaic virus*
Totflow	Mean total number of flower heads from C1, C2 and C3
Totwt	Mean total dry weight (g)
ToYSV	*Tomato yellow spot virus*
TSR	Travelling Stock Reserve
UV	Ultraviolet
V	Valine
V-	Virus free
V+	Virus infected
Vic.	Victoria
WClMV	*White clover mosaic virus*
WMV	*Watermelon mosaic virus*
WNS	Wetland of National Significance
ZYMV	*Zucchini yellow mosaic virus*

GLOSSARY

Ecotype	A distinct group within a species that is adapted to specific habitat conditions
Enemy release	Increase in the impact of an invasive species due to reduced attack by natural enemies
Genetic diversity	The genetic characteristics that constitute a population
Genetic plasticity	The potential for genetic adaptation
Genotype	The genetic composition of an individual
GM_insert	The RNA3 coat protein sequence used for transgenic *Trifolium repens*
Mesic	A moist environment or an habitat with a readily accessible water supply
Naturalised	Non-native species that becomes wild (the population is self-sustaining)
Non-persistent aphid transmission	The virus only remains viable in the vector for a short time
Non-synonymous polymorphism	A nucleotide change resulting in an amino acid replacement
Non-target	An environment where a transgenic plant is not intended to be released
Pathogenicity	The ability of a pathogen to cause damage to a host
Phenotype	The morphology of an individual; the observable traits of an organism

Plant pathosystem	A biological environment consisting of a host and parasite; a term generally used for the types of parasites that spend most their lifecycle inhabiting the host
Polymorphic site	A nucleotide that is a point of mutational variability
Sequence identity	Percent identity; quantification of the similarity between two sequences
Stolon	An above-ground shoot growing parallel to the ground
Variant	Unique sequence
Viral reassortment	The mixing of the genetic material of a species
Viral recombination	The genetic exchange between viruses

1 Introduction

1.1 Biotechnology as a tool for virus resistance

The impact of disease on domesticated plants has necessitated continual endeavours by farmers and plant breeders to find or breed new cultivars with resistance or tolerance to disease (Bosch *et al.*, 2006). In the past the only method of introducing genetic diversity into plants was sexual hybridisation (Melchers and Stuiver, 2000). Genes for resistance could be gained from breeding within existing germplasm (Lin *et al.*, 2007), but the germplasm available to the breeder was limited to species with which the crop was sexually compatible and in many cases resistance remained undiscovered or unavailable in the existing gene pool (Melchers and Stuiver, 2000). More recent methods to introduce genetic variation into crops include mutation by chemicals or radiation and the creation of novel hybrids by cell fusion (Raybould and Gray, 1993). Results from these methods are not as precise as the most recent technique developed: genetic modification (GM).

Genetic modification involves the insertion and integration of foreign DNA into the target species' genome (Raybould and Gray, 1993). An additional benefit of GM is that DNA used for this process can be utilised from any source (biological or artificial), so the germplasm available to plant breeders is practically limitless (Raybould and Gray, 1993, Melchers and Stuiver, 2000). This technology provides the potential to release crops from disease pressure and therefore offers the promise of improving the efficiency of agricultural systems. However, disease resistance is not entirely free of risk, since these altered plants may pose a risk to native plant communities (non-target environments).

1.2 Ecological risks posed by disease-resistant plants to non-target environments

A significant risk posed by disease-resistant plants is the potential for increased weediness or invasiveness of host populations following relief from pathogen pressure (Godfree *et al.*, 2007), a process known as enemy release (Keane and Crawley, 2002).

Evaluating the ecological risks that disease-resistant plants may pose to non-target communities is difficult (Dale *et al.*, 2002), especially given that our knowledge of the plant traits that contribute to weediness is limited, even after decades of observation of the growth of plants in new environments (Hulme, 2009, Browne *et al.*, 2007). Further complexity is added when assessing the risk of plants that have been bred for both persistence (e.g. trees, turf and pasture species) and pathogen resistance.

For any plant with resistance to a pathogen, one or more of the following criteria may indicate an ecological risk to non-target plant communities:
1) naturalisation of the plant in native plant communities; 2) the presence of the disease causing pathogen in native plant communities; 3) the ability of the plant to hybridise with native or naturalised plants; or 4) resistance to the pathogen confers a competitive advantage to the plant. Other factors that also deserve consideration include the heritability of the resistance trait (Conner *et al.*, 2003a) and evolutionary considerations such as a history of co-evolution of the plant and the pathogen, as these factors may influence the durability of pathogen resistance.

Investigation of the relevant risk criteria listed above can provide an understanding of the risks posed to native plant communities by pathogen-resistant plants. This thesis examines criteria 1, 2 and 4 for *Alfalfa mosaic virus* (AMV) and white clover (*Trifolium repens* L.), contributing to an ecological risk assessment of AMV-resistant genetically modified (GM) *T. repens* in Australia. Criterion 3 is not explored as the hybridisation of commercial *T. repens*

cultivars and naturalised *T. repens* ecotypes is highly likely due to the biology of white clover (see section 1.4.1.1 Biology).

1.3 Genetically modified virus-resistant *Trifolium repens*

Alfalfa mosaic virus is endemic in *T. repens* pastures grown in South Eastern (SE) Australia (Norton and Johnstone, 1998, McKirdy and Jones, 1997, McKirdy and Jones, 1995, Coutts and Jones, 2002, McLean, 1983) and is thought to cause major economic losses to the dairy industry (Garrett, 1991). To combat yield losses resulting from viral infection, transgenic virus-resistant *T. repens* genotypes have been developed for future commercialisation in Australia (Spangenberg *et al.*, 2001). In these newly developed *T. repens* genotypes the expression of the viral AMV-coat protein (CP) gene reduces the susceptibility of *T. repens* to the pathogenic effects of AMV. It is envisioned that the commercialisation and release of AMV-resistant *T. repens* in Australia has the potential to ease economic pressures on dairy farmers caused by existing losses in pasture production due to AMV infection, improve animal productivity and reduce the spread of AMV to other pasture legumes that are economically important to the Australian dairy industry.

1.4 Pathosystem

1.4.1 Host: White clover

Trifolium repens is an introduced prostrate perennial legume that is commonly cultivated and known to be naturalised in SE Australia (National Herbarium of New South Wales, 2009). There are no native *Trifolium* species in Australia. Ecotypes of *T. repens* were originally introduced to Australia by early European settlers (Williams, 1987).

1.4.1.1 Biology

Trifolium repens is an obligate outbreeder and has a highly developed genetic gametophytic self-incompatibility mechanism, although a small number of plants are self-compatible. Seed set can be as low as zero seeds per flower head

if the transfer of pollen from anthers to stigmas by outside influences is prevented. If florets are artificially self-pollinated then the amount of seed produced can increase up to an average of 5.7 seeds per flower head (Thomas, 1987). Cross pollination of *T. repens* is thus vital for significant seed production. In natural conditions, bees are primarily responsible for cross pollination (Thomas, 1987). Temperature is an important factor in pollination, pollen germination and pollen tube growth. *Trifolium repens* florets are more attractive to honey bees at temperatures of 27°C or more and that pollen germination and pollen tube growth is faster at higher temperatures (Thomas, 1987).

Trifolium repens seed can be dispersed in a number of ways: Long distance dispersal occurs predominantly in the digestive tracts of grazing animals and birds; seeds can also be spread intermediate to short distances by stock movement, wind, earth worms and dehiscence (Harris, 1987). The durability of *T. repens* seed is influenced by temperature and aeration, however, seed has been shown to remain dormant in soil for up to 46 years (Harris, 1987). Germination of *T. repens* seed can occur at temperatures as low as 5oC, but growth is limited at lower temperatures. The optimum temperature for *T. repens* growth is 24oC (Hart, 1987). In contrast, *T. repens*, once cold hardened, can tolerate temperatures below freezing with minimal tissue damage to the plant (Hart, 1987).

Some properties that enable *T. repens* to thrive in mesic plant communities include: a horizontal leaf arrangement which aids in rapid light interception; the ability to adjust petiole length to compensate for canopy changes and the capacity to proliferate by stolon growth (Harris, 1987). The proliferation of *T. repens* by the development of stolons is very important for the survival of *T. repens* in difficult conditions, especially if flowering and seed production are low (Hart, 1987). *Trifolium repens* is not as tolerant of water stress as other introduced legumes such as lucerne (*Medicago sativa* L.). In response to water stress *T. repens* does not control leaf water loss effectively compared to other

species. In times of drought *T. repens* has been found to have reduced nitrogen fixing ability, lower cuticular resistance to desiccation and slower closing of stomata than *Trifolium subterraneum* or lucerne (Hart, 1987, Crush, 1987). There are also considerable intraspecific variations in the capacity of *T. repens* to survive under nutrient stress. Important factors that influence *T. repens* survival include soil phosphorous and competition, particularly with grasses that are adapted to low nutrient soils (Dunlop and Hart, 1987). Research performed in Wales indicated that soil calcium, phosphorous and pH levels can influence the microdistribution of *T. repens* in communities where conditions for *T. repens* growth are marginal. It was found that *T. repens* occurrence was generally linked to higher levels of calcium, pH and, to a lesser extent, phosphorus (Williams, 1987).

1.4.1.2 The importance of *Trifolium repens* as a pasture species

Trifolium repens is the most important pasture legume in many temperate regions of the world and is currently one of Australia's most widely grown pasture crops (Bouton *et al.*, 2005). *Trifolium repens* is a highly valued feed for dairy cattle and is considered the most important legume used for pasture in the Australian dairy industry (Mason, 1993, Stockdale and Dellow, 1995).

1.4.1.3 Naturalised *Trifolium repens*

Temperate grasslands are one of the world's most abundant ecosystems. These grasslands occur on all continents except Antarctica. Unfortunately, as temperate grasslands tend to be fertile and occur in areas that have attractive temperatures, human development has encroached faster on these environments than on other ecosystems. Over the past 200 years, the human impacts on native grasslands in SE Australia have been disastrous (Parsons, 1992). It is estimated that over 35% of Victoria was originally native grasslands and grassy woodlands, however evidence suggests that less than 1% of these ecosystems still exist (Parsons, 1992). These remaining high value native grasslands and grassy woodlands are threatened not just by human impact but

also by invasive species. A study by Godfree *et al.* (2004b) found that *T. repens* is now a significant component of temperate grasslands and grassy woodlands of NSW and ACT. However, the extent of *T. repens* invasion in other types of native plant communities, including other types of grasslands and grassy woodlands, remains unknown.

1.4.1.4 Susceptibility to viral disease

When grown as a pasture crop in Australia, *T. repens* is susceptible to a number of viral diseases including *Clover yellow vein virus* (ClYVV), *White clover mosaic virus* (WCMV) and *Alfalfa mosaic virus* (AMV) (Latch and Skipp, 1987). Indeed, in 1991 it was estimated that losses in milk production in the Victorian dairy industry due to virus infection of clover exceeded A$20M (Garrett, 1991).

1.4.2 Pathogen: Alfalfa mosaic virus

AMV is a positive-sense single stranded RNA virus from the family *Bromoviridae*, genus *Alfamovirus*. The genome is tripartite with a fourth subgenomic CP mRNA (Hull, 2002). Most legumes are vulnerable to AMV. In the majority of countries where *T. repens* is grown it has been found to be infested with AMV. However, the percentage of plants infected in agricultural areas can be low compared to other viruses (McLaughlin, 1992). Pastures in the USA have been found to contain up to 24% infected plants (Latch and Skipp, 1987). Previous glasshouse studies indicate that AMV can reduce growth of commercial *T. repens* cultivars by up to 33% compared to healthy plants (Gibson *et al.*, 1982, Gibson *et al.*, 1981, Miller, 1962, Houston and Oswald, 1953). Signs of infection include vein clearing and yellow mottling of young leaves. As symptoms develop, angular yellow patches or necrotic lesions can occur on the leaves and general growth of the plant is stunted. Symptoms are most noticeable at temperatures ranging from 18 to 24°C (Latch and Skipp, 1987). Infected plants can be impacted by having fewer leaves, stolons and rooting nodes, flower heads and resultant seed production, reduced nodulation, and reduced cold tolerance (Latch and Skipp, 1987). Previous

studies indicate that AMV is easily transmitted to *T. repens* mechanically and by a number of aphid species in a non-persistent manner (Latch and Skipp, 1987, Johnstone and Chu, 1993). Dodder (*Cuscuta gronovii)* has also been found to be responsible for transmission. AMV is transmitted by seed and pollen in some hosts but this has not been found in *T. repens* (Latch and Skipp, 1987). Although AMV is a well-studied virus (Barker *et al.*, 1983, Cornelissen *et al.*, 1983, Cornelissen, 1983), no work has yet been undertaken to study the effects of AMV on naturalised *T. repens* or the diversity of Australian AMV genotypes.

1.5 Concerns regarding transgenic virus-resistant *Trifolium repens*: the biology of *Alfalfa mosaic virus*

Although many recent technical advances have been made in the area of molecular biology, studies regarding the evolution, variability and genetic structure of plant viruses, and the influence of plant viruses on host biology are still relatively rare (García-Arenal *et al.*, 2001). There are three key concerns regarding AMV and the intentional environmental release of transgenic *T. repens* in SE Australia as little is known regarding the genetic diversity or population ecology of AMV in this region. Firstly, will transgenic plants be resistant to SE Australian AMV? Secondly, how durable is the resistance trait? And thirdly, will release of the transgenic result in the emergence of novel, disease-causing, viruses?

1.5.1 Resistance of the transgenic

There is a high possibility for genetic variation in viruses compared to other organisms, due to factors such as increased rates of mutation; large population sizes; and the ability to undergo recombination and reassortment (García-Arenal *et al.*, 2001). In addition, many plant viruses cause persistent infections in their hosts, and their populations can reach very large sizes within a plant (García-Arenal *et al.*, 2001).

Although AMV is a well-studied virus internationally, little is known regarding the genetic diversity of Australian AMV and it is not known how similar the CP sequence used to confer AMV resistance in transgenic *T. repens* is to the CP of AMV in SE Australia. Any sequence diversity of Australian AMV genotypes compared to the CP used for the transgene may result in AMV overcoming resistance. Taschner *et al*. (1994) provided evidence that a CP transgene sourced from an AMV mutant, with one amino acid change, was unable to provide resistance against wild-type AMV.

In addition, complementation, the process by which deleterious mutants may be aided by the function of fully intact genotypes (García-Arenal *et al.*, 2001), has been illustrated for AMV. It has been demonstrated that for cell-to-cell movement AMV is able to utilise movement proteins from other members of the family Bromoviridae (Sanchez-Navarro *et al.*, 2006). CPs from alfamoviruses and ilarviruses can be interchanged resulting in successful binding and genome activation (Tenllado and Bol, 2000). It is possible that deleterious mutant viruses exposed to the AMV coat protein in the transgenic *T. repens* genome may gain full functionality, resulting in an increase in the effective population size and possibly an increase in genetic diversity of the virus population.

1.5.2 Durability of resistance

It is currently unknown how durable AMV CP-mediated resistance would be for *T. repens.* Plant resistance to viral diseases can be produced through various mechanisms. Each of these mechanisms may place the virus under different selection pressures (Bosch *et al.*, 2006). Bosch *et al.* (2006) found that resistance that is facilitated by the vector, or resistance resulting in a reduction in the inoculation of the plant does not place the virus under selection pressure to evolve an increased multiplication rate. In contrast, within-plant resistance, including transgenic resistance such as AMV resistance in *T. repens*, facilitating

a reduction of virus titre or a reduction in symptoms puts the virus under selection pressure to evolve an increased multiplication rate and may result in an increase in genetic diversity of the virus population.

Selective pressures from the host's defences can be a significant influence on pathogen evolution (Wilson *et al.*, 2005). Selection can result in a reduction in population diversity, and can result in increased differentiation between populations (French and Stenger, 2003). Selection pressures may include: maintenance of structural features of the virus and viral-genome; resistance genes; virulence; and vector-associated selection (García-Arenal *et al.*, 2001). Host resistance may lead to the evolution of more aggressive virus types (Bosch *et al.*, 2006).

1.5.3 Emergence of novel virus species

Recent advances in molecular ecology have provided clues regarding novel virus emergence, although emergence continues to be inadequately understood. Novel virus emergence appears to be multifactorial, resulting from an assortment of changes in the environment, pathogen, host, and vector (Fargette *et al.*, 2006). The challenge is understanding host-pathogen (H-P) interactions in order to better predict the risk of emergence of novel virus species or more virulent strains.

Fargette *et al.* (2006) describes three stages of viral emergence: firstly, host range expansion occurs; secondly, there is spread of the pathogen; and thirdly, there is an increase in the occurrence of infection. Crucial factors that can cause novel virus-vector-plant-environment interactions include viral recombination, new vectors or changes in vectors, and host interactions such as genome integration and adaptation. Long distance dispersal mechanisms can also ultimately result in new virus emergence (Fargette *et al.*, 2006). These factors can be influenced by agricultural changes.

The emergence of some new viral species such as *Rice yellow mottle disease* have been strongly linked to agricultural advancement (Fargette *et al.*, 2006). An example of emergence, *Banana streak virus*, is thought to be a result of the interaction between wild African monocots and introduced species such as maize and sugarcane (Fargette *et al.*, 2006). Gibbs *et al.* (2008) found that a major divergence of potyvirus occurred approximately 6,600 years ago during a period when many plants were domesticated for agriculture, and the movement of potyviruses to Australia may have been facilitated by the Austronesian people when they brought domesticated plants to the region approximately 2000 years ago.

As altering the host changes the pathosystem (Fargette *et al.*, 2006), the introduction of transgenic *T. repens* would result in a change in the pathosystem, how Australian AMV would respond is currently unknown.

1.6 Concerns regarding transgenic virus-resistant *Trifolium repens*: the biology of *T. repens*

Transgenic virus-resistant *T. repens* has been identified as a potential source of risk to non-target plant communities in Australia, due to the ecology, distribution and population dynamics of *T. repens*. Firstly, *T. repens* has been bred to persevere with very little intervention in a range of environments, and is known to be a weed in some temperate regions of Australia characterised by moderate to high annual rainfall (Godfree *et al.*, 2004b, Godfree *et al.*, 2004a). Secondly, *T. repens* is an obligate cross-pollinating species (Thomas, 1987), indicating that gene flow between commercial and naturalised *T. repens* genotypes is likely. Thirdly, AMV is known to be prevalent in *T. repens* pastures in Australia (Norton and Johnstone, 1998), and it has been detected in a number of temperate grasslands and grassy woodlands in SE Australia (Godfree *et al.* 2004b). This suggests that AMV may potentially play a role in limiting *T. repens* populations in Australian native plant communities, and that the release of virus-resistant genotypes could pose a threat to high

conservation-value plant communities in this region. However, the extent of AMV in naturalised *T. repens* in most plant communities remains unknown.

If virus resistance is found to confer a fitness advantage then any gene flow between GM *T. repens* and naturalised populations of *T. repens* could result in considerable ecological, economic and trade implications for Australia. Therefore it is critical that a rigorous ecological risk assessment is conducted to determine whether AMV resistant GM *T. repens* is safe to be grown as a fodder crop in an Australian environment.

1.7 Transgenic regulatory structure in Australia

In 2000 the Australian Government passed the *Gene Technology Act 2000* (Office of Legislative Drafting and Publishing, 2006). This Act was created to establish a national regulator responsible for the licensing and release of GM organisms. The *Gene Technology Act* is consistent with the precautionary principle, whereby if there are threats of severe or irreparable environmental degradation, a deficit of scientific information should not be used as an excuse for delaying cost-effective methods to prevent environmental damage. A regulatory agency, the Office of Gene Technology Regulator (OGTR), overseen by the Gene Technology Regulator (GTR), administers the *Gene Technology Act 2000* and makes regulatory decisions relating to gene technology use across Australia (Office of Legislative Drafting and Publishing, 2006).

Although plant breeders are increasingly using targeted breeding or biotechnology to produce plants with resistance to plant pathogens (Gu *et al.*, 2008, Jauhar, 2006), gene technology is viewed as a controversial technique for plant improvement in Australia (Stanley *et al.*, 2003, Linacre *et al.*, 2006). Internationally it is recognised as a powerful technology that offers potentially enormous benefits, but it is also recognised that it is not without risks (Alston, 2004). The GTR must assess any potential risks a GM crop may pose to the environment or to the health and safety of people before it can be approved for

release. Factors to be considered include the effect of the identified modification, provisions for limiting the persistence of the GM crop in the environment and the extent of the proposed release. Potential risks, relevant to *T. repens*, considered as part of a risk assessment include: the risk of genes from the GM plant moving to an existing weed species; the extent to which genes can transfer from the GM crop to other non-GM crops; and the degree to which targeted pathogenic organisms may in the future become resistant to the new technology.

Trifolium repens is distinct from many of the plants which have progressed through the Australian GM regulatory system, for example cotton, carnation and canola (Office of the Gene Technology Regulator, 2006, Office of the Gene Technology Regulator, 2002a, Office of the Gene Technology Regulator, 2002b). Many of these plants are crops that no longer possess the weedy attributes that may have been present in their wild relatives, therefore their ability to become weeds has been significantly impeded (Conner *et al.*, 2003a). Common weedy characteristics lost include phenotypic plasticity, seed dormancy, variable growth, frequent flowering and seed production, and seed dispersal methods (Conner *et al.*, 2003a). Therefore these crops are unlikely to become weedy, unlike *T. repens*. It is recognised that crops such as legumes and grasses, due to a short history of domestication, are more likely to become weedy (Conner *et al.*, 2003a).

1.8 Risk analysis framework

Methods for environmental risk assessments are prescribed in regulatory documents such as the Cartagena Protocol (Secretariat of the Convention on Biological Diversity, 2000) and the European Commission Directive 2001/18 (The European Parliament and of the Council, 2001). Past ecological risk assessments often centre on gene flow to wild relatives or impacts on species used as food (Firbank *et al.*, 2005). Conner *et al.* (2003a) expresses risk as:

Risk = probability X consequence

= likelihood of event X (negative) impact of event

In general, risk assessment aims to answer three questions:

1. What possible harm can occur?
2. What is the likelihood of harm occurring?
3. What are the costs if harm occurs?

Conner *et al.* (2003a) suggests an additional question:

4. What will be the costs if the GM plant is disallowed?

If transgenic *T. repens* is released then gene flow to naturalised *T. repens* is likely, therefore the predominant concern for this risk assessment is that the transgene may confer a fitness advantage to naturalised *T. repens* (risk assessment question one above) (Godfree *et al.*, 2007). This risk is commonly considered for GM crops, as the transgene may provide a selective advantage to naturalised relatives, resulting in populations of naturalised relatives becoming more invasive following relief from pathogen pressure [the enemy release hypothesis (Keane and Crawley, 2002)]. Evaluating the risks that disease-resistant plants may pose to non-target plant communities is challenging (Dale *et al.*, 2002). Indirect effects due to changes in land management practices resulting from the uptake of GM crops have been documented (Firbank *et al.*, 2005). However, so far there is little documented evidence of direct environmental risks to non-target ecosystems arising from the commercial release of GM plants (Firbank *et al.*, 2005).

This thesis aims to address the ecological aspects of risk assessment questions two and three (see above) by determining the distribution of *T. repens* and AMV in non-target plant communities and assessing the ecological impact of GM-conferred AMV-resistance in *T. repens*.

A critical component of an ecological risk assessment is defining a relevant baseline for evaluation and decision making. In most cases a suitable reference point is a plant that has been produced through traditional breeding methods (Conner *et al.*, 2003a). If the desired trait can be bred conventionally then a plant with the GM-conferred trait is likely to have similar impacts as a plant with the trait bred through conventional breeding methods. This is the ideal comparison, as it is likely that land management practices for the GM and traditionally bred plants are identical. However, this situation is rare, as many desired GM-conferred traits are unable to be bred conventionally, so depending on the trait a suitable reference point is required. In this study I used commercial and naturalised *T. repens*, as these are likely to eventually contain the transgene conferring resistance to AMV.

1.9 Thesis aims and structure

1.9.1 Aims

The primary aim of this thesis was to complete the initial stages of the ecological risk assessment of transgenic AMV-resistant *T. repens* in SE Australia, with a focus on determining the potential for increased weediness of non-target populations of *T. repens* following the release of commercial GM virus-resistant lines. This thesis involved four key stages: 1) identification of at-risk high conservation-value native plant communities and environments in NSW, Vic. and the ACT, 2) quantification of the abundance and distribution of both *T. repens* and AMV in non-target environments, 3) quantification of the genetic diversity of AMV populations from across the study region, as an indicator of adaptive/ evolutionary potential of the virus, and 4) assessment of the infectivity and aggressiveness of a range of representative AMV isolates.

This thesis provides comprehensive data on the distribution and abundance of *T. repens* and AMV in non-target communities across NSW, the ACT and Vic., the diversity and evolutionary background of AMV isolates from these regions, assessment of the factors predisposing native plant communities to risk of

invasion by AMV-resistant *T. repens*, and assessment of the pathogenicity of AMV collected from naturalised *T. repens* populations.

1.9.2 Structure

This thesis describes the results gained from the ecological risk assessment of GM AMV-resistant *T. repens*. This thesis was conducted as a collaborative PhD project involving the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Plant Industry, Dairy Australia and The Australian National University (ANU). The thesis was supervised by Dr. Robert Godfree (CSIRO) and Dr. Celeste Linde (ANU).

This thesis has been produced with the aim of each data chapter (Chapters 2-4) being a stand-alone published manuscript. Although the content is specifically tailored to suit individual chapters there is some repetition and similar themes in the introductions and discussions of these chapters, the general introduction (Chapter 1) and the synthesis of the thesis (Chapter 5). Methods relevant to individual chapters are contained within that chapter.

Chapter 2 examines the prevalence of naturalised *T. repens* and associated pasture viruses in non-target plant communities in SE Australia. That chapter includes a description of the composition of communities, the distribution and the abundance of *T. repens*, the incidence of pasture viruses within the communities visited and the implications for the risk assessment of transgenic virus-resistant *T. repens*.

Chapter 3 explores the genetic diversity of AMV present in naturalised *T. repens* in SE Australia, the distribution of different AMV genotypes across the landscape, and the likely source of SE Australian AMV. That chapter examines the evolutionary potential of AMV in this region to overcome CP-conferred resistance in GM *T. repens* and evaluates the environmental implications of transgenic clover release.

Chapter 4 investigates the virulence and infectivity of AMV isolates from naturalised *T. repens* populations and considers the likely impacts of AMV on the growth and survival of a selection of the populations of naturalised *T. repens* investigated in Chapter 2 and commercial *T. repens* cultivars. The degree of environmental risk posed by AMV-resistant transgenic *T. repens* to non-target habitats in NSW, the ACT and Vic. is also considered.

Finally, Chapter 5 of the thesis combines all outcomes of the thesis in a general synthesis. This chapter provides a summary of the thesis results, the final conclusions and infers the degree of environmental risk posed by AMV-resistant transgenic *T. repens* to non-target plant communities in parts of NSW, the ACT and Vic. Insights from my study relevant to the general management and risk assessment of transgenic pasture species are also explored.

2 Geographic distribution of three viruses in naturalised *Trifolium repens* and implications for a risk assessment of a genetically modified *Alfalfa mosaic virus*-resistant *T. repens*

2.1 Introduction

Plant breeders and researchers are increasingly utilising targeted breeding or biotechnology to produce pathogen-resistant (PR) plants (Gu *et al.*, 2008, Jauhar, 2006) that have the potential to improve the efficiency and productivity of agricultural systems. However, some PR plants pose a potential threat to non-target ecosystems that lie beyond the scope of the intended commercial release, since disease-resistant genotypes may exhibit increased weediness or invasiveness of host populations following relief from pathogen pressure, a process known as enemy release (Keane and Crawley, 2002). Indeed, it has recently been shown that increased population growth rates and niche expansion of host populations in non-target areas may occur following introgression of disease resistance genes from genetically modified (GM) virus-resistant plants (Godfree *et al.*, 2007, Godfree *et al.*, 2009a, Godfree *et al.*, 2009b).

However, evaluating the risks that disease-resistant plants pose to non-agricultural ecosystems remains a daunting challenge (Dale *et al.*, 2002). Apart from the fact that our knowledge of the plant traits that contribute to weediness is limited, even after decades of observation on the movement of plants to new environments (Hulme, 2009, Browne *et al.*, 2007), the specific role that diseases play in limiting the spatial distribution and abundances of plant hosts is in most cases unknown, apart from a few well-documented cases involving catastrophic diseases caused by pathogens such as *Cryophonectria parasitica* (Paillet, 2002) and *Phytophthora cinnamomi* (Shearer *et al.*, 2008). Typically, however, the impacts of disease on host populations are likely to be

more subtle, and interact with factors such as habitat type (Godfree *et al.*, 2009b), host density (Ferrandino, 2005), host-pathogen (H-P) coevolutionary dynamics (Fargette *et al.*, 2006, Jones, 2006), and heritability of resistance traits (Conner *et al.*, 2003b).

The development of PR plants that target multi-disease pathosystems (e.g., Bt cotton (Benedict *et al.*, 1996)) pose a significant new challenge to ecologists engaged in risk assessment. Multi-species pathosystem complexes are common in nature (Raybould *et al.*, 2002, Xu *et al.*, 2008), and the presence of spatial and temporal variation in disease incidence across species (García-Arenal *et al.*, 2001), along with competitive, compensatory mechanisms or symptom enhancement among pathogens results in strong interactions between different diseases and their hosts (Alves-Júnior *et al.*, 2009, Xi *et al.*, 2007). While some studies to date have investigated the risk of ecological release of PR plants focused on single H-P systems (e.g., Godfree *et al.* (2009a,b)), the recent development of transgenic plants that express resistance to a multiple pathogens by methods such as marker assisted breeding (i.e. bean cultivars resistant to anthracnose, angular leaf spot and rust (Ragagnin *et al.*, 2009)), multiple pathogen derived transgenes (i.e. squash resistant to *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* (WMV), *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ring spot virus* (PRSV) (Silora *et al.*, 2006)) or RNA silencing (i.e. *Nicotiana benthamiana* resistance to four tospovirus species (Bucher *et al.*, 2006)), pose a significant new challenge to ecologists engaged in risk assessment. This is especially true of pasture plants which add an additional challenge, as they have a track record of invasiveness in non-target ecosystems (Lonsdale, 1994). Some research underpinning risk assessment of pasture species has been undertaken (Wang *et al.*, 2004, Cunlife *et al.*, 2004, Kang *et al.*, 2009), however very few ecological risk assessments have been completed for transgenic pasture species (Bagavathiannana and Van Ackerb, 2010, Sandhu *et*

al., 2008, Sandhu *et al.*, 2009) and none to date for GM pasture species resistant to multiple pathogens.

This chapter reports the initial stages of the ecological risk assessment of transgenic *Trifolium repens* (white clover) genotypes that express resistance to *Alfalfa mosaic virus* (AMV) (Spangenberg *et al.*, 2001). *Trifolium repens* is a well-studied model GM pasture species (Godfree *et al.*, 2007, Godfree *et al.*, 2006, Godfree *et al.*, 2009a) which meets several criteria indicative of potential risk to non-target plant communities. In Australia, *T. repens* is known to be naturalised or invasive in some native plant communities (Godfree *et al.*, 2004a) and can be infected by numerous viral diseases (Norton and Johnstone, 1998, McKirdy and Jones, 1997, McKirdy and Jones, 1995, Coutts and Jones, 2002, McLean, 1983). Consequently, it has been argued niche expansion following release from one such pathogen (ClYVV (Godfree *et al.*, 2009a)) would likely occur if resistance genes from newly developed PR genotypes, were to enter non-target populations, thus posing a potential threat to some high conservation value native plant communities. Given *T. repens* is the most important pasture legume in many temperate regions of the world and is currently one of Australia's most widely grown pasture crops (Bouton *et al.*, 2005), an ecological risk assessment of any GM genotype of this species is important prior to environmental release.

Here, I apply a simple framework for assessing the risk posed by PR species potentially maintaining large populations in non-target habitats over extensive geographic areas (Figure 2.1). In this framework, the early stages of risk assessment involve habitat identification, where potential habitats for further detailed study are identified, followed by field surveys, where information on the distribution and abundance of pathosystem components is collected. These data are then used to inform the development of the next stages of the tiered experimental risk assessment (see Wilkinson and Tepfer (2009) for discussion

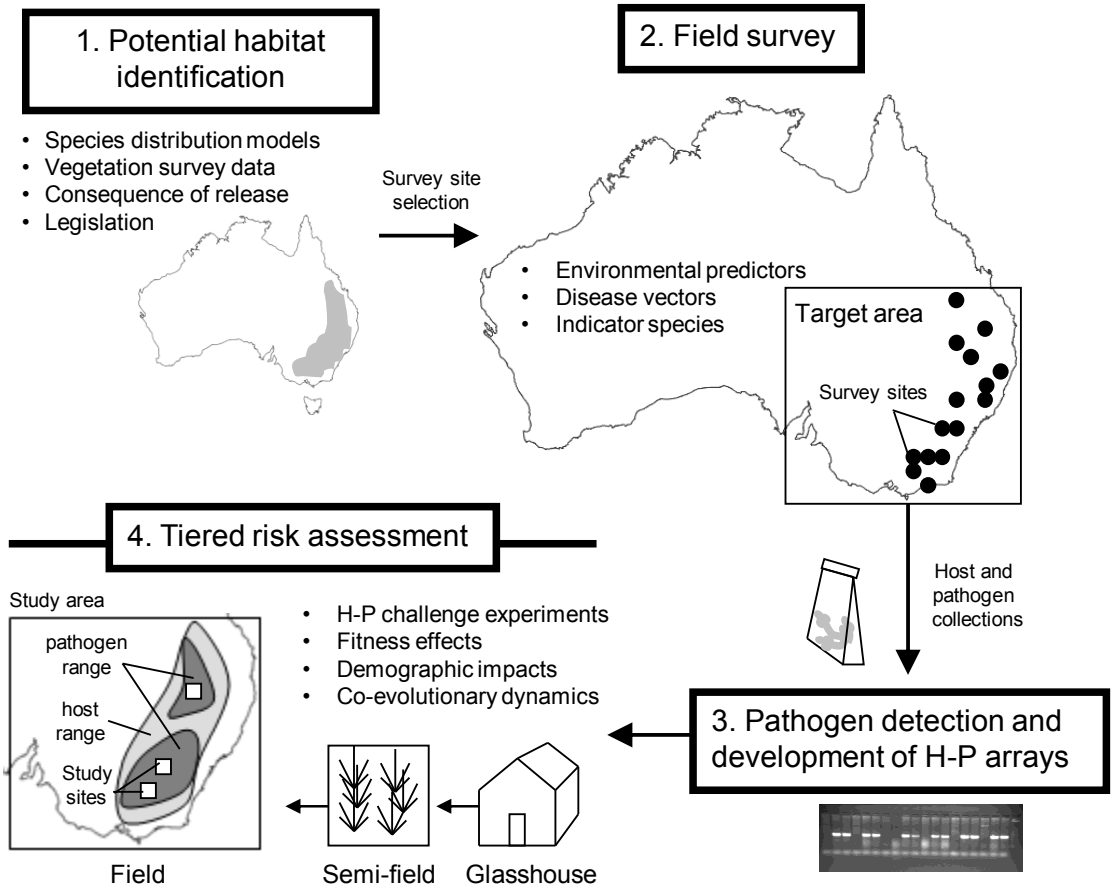


Figure 2.1: Schematic illustrating a procedure for assessing the potential risk posed by pathogen-resistant plants to non-target ecosystems at large spatial scales. Identification of potential habitat (Stage 1) and field surveys in the target area (Stage 2) are key to generation of representative host-pathogen (H-P) arrays (Stage 3). These arrays are then used in controlled H-P challenge experiments and *in situ* demographic field studies targeting at-risk habitats (Stage 4). Details concerning the implementation of tiered risk assessment strategies are provided in Wilkinson and Tepfer (2009) and Godfree *et al.* (2009a,b).

of tiered risk assessment procedures, and Godfree *et al.* (2009a,b) for application). A significant element of this framework involves critical decision-making early in the risk assessment process. Herbarium records, vegetation data and species distribution models (Hill, 1996) are used to identify potential host habitat types. Habitats are then prioritised based on government conservation priorities (Office of Legislative Drafting and Publishing, 2007, Victorian Government, 2007, New South Wales Government, 2008). Clearly, as the complexity of the pathosystem increases, and the geographic distribution of the host species increases, the field survey component of the work can become large.

The key aim of this study was to examine the spatial distribution and abundance of *T. repens* and a range of co-existing viral pathogens, AMV, *White clover mosaic virus* (WClMV) and ClYVV in multiple potentially at-risk habitat types across a 300,000 km^2 region of south-eastern (SE) Australia (Figure 2.1: stages 1 and 2). This data will then be used to identify at-risk non-target environments and relevant pathosystems on which to base future tiered risk assessment efforts. Although the focus of the risk assessment is AMV-resistant *T. repens*, which is the most immediate GM virus-resistant pasture plant being assessed for commercial release in Australia (Office of the Gene Technology Regulator, 2009a, Office of the Gene Technology Regulator, 2009b, Office of the Gene Technology Regulator, 2009c), the structure and distribution of ClYVV- and WClMV-*T. repens* pathosystems, and the extent of co-infection in non-target host populations is also considered. The specific objectives were to:

i. Identify potential non-target habitats that may be placed at risk by the release of virus-resistant T. repens;

ii. Determine the distribution and abundance of *T. repens* and co-existing viral pathogens (AMV, ClYVV, WClMV) in non-target habitats

(including endangered ecosystems) that occur in a range of bioregions in SE Australia;

iii. Identify the geographic and site-level factors that determine the distribution and abundance of *T. repens* and associated viruses in the study region;

iv. Identify, based on the spatial distribution of host-virus pathosystem components, potentially at-risk non-target ecosystems;

v. Investigate the experimental methods required to refine the experimental component of the tiered risk assessment (Figure 2.1: stage 3).

I also draw on the results to consider the implications that geographic variability in the structure of multi-species pathosystem complexes have for the risk assessment of PR plants in general. To my knowledge this is the largest study conducted to assess the ecological implications associated with the release of PR genotypes into a pathosystem complex that occurs in multiple bioregions at the continental scale. As the study required refinement of the understanding of the link between pathosystem characteristics (vegetation type, abundance of *T. repens*, site location and climate), land management regimes and epidemiology, my results have broad implications for risk assessment of PR transgenic plants on a global basis.

2.2 Methods

2.2.1 Selection of non-target habitats for Trifolium repens and virus surveys

A total of 213 survey sites were selected in which to assess *T. repens* abundance and virus frequency within a 300,000 km2 study area in SE Australia (Figure 2.2a). Sites were present in 36 plant potential habitat types that occurred within

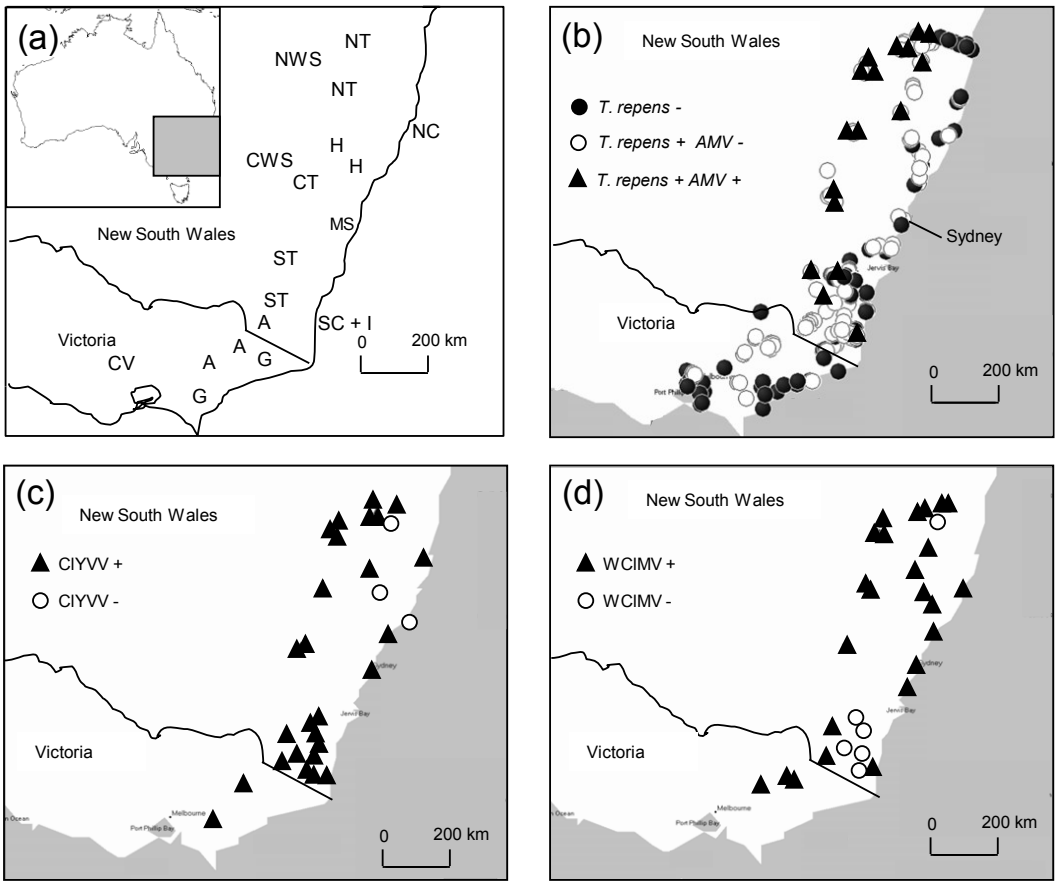


Figure 2.2: Location of survey sites in south-eastern Australia. (a) geographic regions referred to in NSW and Victoria; insert shows position of general study region within Australia. (b) position of all survey sites containing *Trifolium repens* and *Alfalfa mosaic virus* (AMV). (c) distribution of *Clover yellow vein virus* (ClYVV) based on sites tested for ClYVV. (d) distribution of *White clover mosaic virus* (WClMV) based on sites tested for WClMV. In (a) NWS = Northwest Slopes, NT = Northern Tablelands, CWS = Central West Slopes, H = Hunter Valley and Barrington regions, NC = North Coast, CT = Central Tablelands, MS = Metropolitan Sydney, ST = Southern Tablelands, A = alpine and subalpine region (NSW and Victoria), SC = South Coast and Illawarra, G = Gippsland Victoria, CV = Central Victoria.

the climatic envelope for *T. repens* (Hill, 1996); most habitats had high conservation value (listed as threatened or endangered at state or federal level), were within the region where commercial release of PR *T. repens* genotypes is likely, or were likely to contain large virus-infested populations of *T. repens* to act as a source of viral inoculum (e.g., roadsides). The list of potential habitats was determined based on literature describing the flora of SE Australia, identification of endangered or threatened plant communities, and consultation with relevant government bodies (e.g., the OGTR) involved in the risk assessment process. Survey sites were widely distributed across SE Australia (Figure 2.2a) and occurred in a range of geographic regions and bioregions that delineate dominant vegetation types across the study region (Table 2.1). Most importantly, the survey included 21 plant communities threatened or endangered at the national or state level, a range of communities occurring in Wetlands of National Significance (WNS), and numerous sites in National Parks (NP) (Table 2.1). Relevant references for all habitats and regional plant communities are provided in Appendix 2.1.

At all surveyed sites a range of parameters for habitat description and quantification of the distribution and abundance of *T. repens* and associated viruses were recorded. These were: location (latitude and longitude), habitat type (see Table 2.1), *T. repens* abundance, disturbance level, distance (nearest km) to closest cropped area (≤ 1 km or > 1 km), and conservation value. *Trifolium repens* abundance was determined based on a semi-quantitative scale containing five classes (0 = absent, 1 = a few plants present, 0-1% cover, 2 = common with 1-5% cover, 3 = abundant, a dominant understory plant with 5-30% cover; 4 = very abundant and approaching a monoculture in many areas with >30% cover overall). Cropping activity in surrounding sites was defined as obvious tillage or cultivated *T. repens* pasture.

The level of disturbance at each site (at the time of sampling) was classified as high, medium or low. Highly disturbed sites were those in which disturbance

Table 2.1: Habitat types surveyed for *Trifolium repens*, *Alfalfa mosaic virus* (AMV), *Clover yellow vein virus* (ClYVV) and *White clover mosaic virus* (WClMV). Categories for conservation status, disturbance level, cropping proximity and *T. repens* abundance are provided in the text. All sites in which *T. repens* was present were tested for the presence of AMV while a subset were tested for WClMV and ClYVV.

Habitat type	Region[a]	Conservation Status[b]	Disturbance level[b]	Crop ≤1km[c]	Sites surveyed	Sites with *T.repens*	Average *T.repens* abundance[d]	Sites with AMV	Sites with WClMV	Sites with ClYVV
Alpine bog, heath and snowpatch										
Alpine Bog Community or Fen (Bog Pool) Community	3	VH	L,M	N	5	1	1.0	0	nt	nt
Alpine heath	9	H	L	N	2	2	4.0	0	nt	nt
Alpine snowpatch community	3	VH	L,M	N	2	2	2.0	0	nt	nt
Coastal and coastal plain forests, woodlands and grasslands										
Northern Warm Temperate Rainforest	4	H	L	N	2	0	-	-	-	-
Bega dry grass forest	4	VH	L	Y, N	6	2	1.0	0	nt	nt
Brogo wet vine forest	4	VH	L	N	3	0	-	-	-	-
Candello dry grass forest in the South East Corner Bioregion	4	VH	H	Y	1	1	2.0	0	nt	nt
Lower Hunter Spotted Gum- Ironbark Forest in the Sydney Basin Bioregion	9,13	VH	L, M	Y, N	5	2	1.5	0	nt	nt
South Coast Sands and Southern Tablelands Dry Sclerophyll Forest	4,9	M, H	L, M, H	Y, N	6	1	1	0	0/1	1/1
Southern Escarpment Wet Sclerophyll Forest remnant	1	VH	L	N	1	0	-	-	-	-
Subtropical Forest	13	H	L	N	1	0	-	-	-	-
Coastal woodland and grassland	1,2,4,5	M, H	L, M	Y,N	10	2	2.5	0	0/1	1/1
Inland lowland, montane and subalpine forests and woodlands										
Montane Wet Sclerophyll Forest	9	M, H	L, M	N	3	3	4.0	0	1/1	1/1
Ribbon Gum, Mountain Gum, Snow Gum Grassy Forest/Woodland of the New England Tableland Bioregion	1,13	VH	L, M	Y, N	3	3	2.3	1	0/1	1/1
New England peppermint woodland on Basalts and Sediments in the New England Tableland Bioregion	7	VH	L	Y	1	1	4.0	1	0/1	1/1
Inland Casuarina riparian woodland	11	M	H	Y	1	1	3.0	1	1/1	1/1
White Box- Yellow Box- Blakely's Red Gum Grassy Woodland and Derived native Grassland	7,11	VH	L, M	Y, N	7	7	3.1	2	2/2	2/2
Subalpine + Montane Woodland	3,9,10,13	M, H	L, M, H	Y, N	24	19	2.2	2	2/5	5/5
Lowland, montane and subalpine grassland										
Central Gippsland Plains Grassland Community	1	M	M, H	N	2	1	1	0	nt	nt
Subalpine sod-tussock grassland	10	VH	L, M	N	6	6	3.7	1	2/2	2/2
Montane grassland	3	H	M	N	1	1	3	0	nt	nt
Natural Temperate Grassland of the Southern Tablelands of NSW and the Australian Capital Territory	9	VH	L, M, H	Y, N	14	11	3.4	2	1/3	3/3
Plains Grassland (South Gippsland) Community	1	VH	L	N	1	0	-	-	-	-
Western (Basalt) Plains Grassland Community	2	VH	L, M, H	N	4	2	2	0	nt	nt

[a]Region 1 = Gippsland Victoria, 2 = central Victoria, 3 = alpine and subalpine Victoria, 4 = south-coastal and Illawarra NSW, 5 = mid- and north-coastal NSW, 6 = metropolitan Sydney, 7 = northern tablelands NSW, 8 = central tablelands NSW, 9 = southern tablelands NSW and ACT, 10 = alpine and subalpine NSW, 11 = northwest slopes NSW, 12 = central west slopes NSW, 13 = Hunter Valley and Barrington region, NSW

[b]VH = very high, H = high, M = medium, L = low

[C]Y = yes, N = no

[d]1= low level, 2= moderate level, 3= abundant, 4= very abundant

nt = not tested

Table 2.1: continued

Habitat type	Region[a]	Conservation Status[b]	Disturbance level[b]	Crop ≤1km[c]	Sites surveyed	Sites with *T.repens*	Average *T.repens* abundance[d]	Sites with AMV	Sites with WCIMV	Sites with CIYVV
Wetlands, swamps, saltmarshes										
Upland wetlands of the New England Tablelands and the Monaro Plateau, Upland Wetlands of the Drainage Divide of the New England Tableland Bioregion	7	VH	L, H	Y, N	2	2	4.0	1	1/1	1/1
Montane peatlands and Swamps of the New England Tableland, NSW North Coast, Sydney Basin, South East Corner, South Eastern Highlands and Australian Alps	3,5,9,13	VH	L, M	N	7	5	2.6	0	0/1	1/1
Fresh water wetlands on coastal floodplains of the NSW North Coast, Sydney Basin and South East Corner	4,6,13	VH	L,M	N	8	2	1.5	0	nt	nt
Herb-rich Plains Grassy Wetland (West Gippsland) Community (Temperate Lowland Plains Grassy Wetland)	1	VH	H	Y	1	0	-	-	-	-
Freshwater Wetlands in the Sydney Basin Bioregion	6	VH	M, H	N	2	0	-	-	-	-
Coastal Saltmarsh in the NSW North Coast, Sydney Basin and South East Corner Bioregions	1,5	M, H, VH	L, M	N	3	0	-	-	-	-
Red Gum Swamp Community	1,2	VH	L, M	Y, N	2	0	-	-	-	-
Sedge-rich *Eucalyptus camphora* Swamp Community	9	VH	L	Y	1	0	-	-	-	-
Coastal Heath + Swamp	1	M, H	L, M	Y, N	3	1	1	0	nt	nt
Wetlands (other)	1,2,4,5,6,9	H, VH	L, M, H	Y, N	15	1	4	0	nt	nt
Roadsides, modified vegetation, stock reserves										
Roadsides, disturbed (coastal, lower elevations)	2,4,5,8,9,11,13	L, M	M, H	Y, N	29	27	3.2	7	7/9	8/9
Disturbed, roadsides (subalpine, alpine)	3,9,10	L, M	M, H	Y, N	14	14	3.4	0	0/1	1/1
Travelling stock reserves with minimal native vegetation	4,5,7,9	L, M, H	L, M, H	Y, N	15	5	1.8	1	1/1	1/1

[a]Region 1 = Gippsland Victoria, 2 = central Victoria, 3 = alpine and subalpine Victoria, 4 = south-coastal and Illawarra NSW, 5 = mid- and north-coastal NSW, 6 = metropolitan Sydney, 7 = northern tablelands NSW, 8 = central tablelands NSW, 9 = southern tablelands NSW and ACT, 10 = alpine and subalpine NSW, 11 = northwest slopes NSW, 12 = central west slopes NSW, 13 = Hunter Valley and Barrington region, NSW
[b]VH = very high, H = high, M = medium, L = low
[C]Y = yes, N = no
[d]1= low level, 2= moderate level, 3= abundant, 4= very abundant
nt = not tested

had severely limited the growth of native species and strongly altered the structure of the plant community; this usually occurred due to severe grazing by livestock, extensive fire or land management practices which altered the physical environment. Sites characterised by medium disturbance contained partially intact native vegetation but with clear evidence of compositional and structural change; usually associated with activities such as light grazing or occasional mowing. Sites with a low disturbance ranking were characterised by minimal recent disturbance and contained largely intact native plant communities.

The conservation value of each site was classified on the basis of four subjective categories arranged in generally declining conservation significance. Categories were:- 1) very high: endangered or threatened plant communities listed within Australia at the state or federal level, WNS and wetlands listed under the Convention on Wetlands of International Importance (the Ramsar Convention); 2) high: sites containing largely intact, minimally disturbed remnant native vegetation within a NP, travelling stock reserve (TSR) or other type of reserve; 3) medium: native vegetation occurring within a NP, TSR, reserves or adjacent to roads but with a moderate to high level of disturbance; and 4) low: heavily disturbed areas and roadside verges (within 5 m of the road edge) with little or no remaining native vegetation.

In addition to the 37 primary habitat types investigated in the study (Table 2.1), sites from across the survey region were also grouped into the following broader habitat types that capture much of the general floristic variation present in SE Australian vegetation:- 1) alpine bog, heath and snowpatch; 2) coastal and coastal plain forest, woodlands and grasslands; 3) inland lowland, montane and subalpine forests and woodlands; 4) inland lowland, montane and subalpine grassland; 5) wetlands, swamps and saltmarshes, and 6) roadsides, heavily modified vegetation, and stock reserves (Table 2.1).

2.2.2 *Trifolium repens collections*

Trifolium repens stolons were collected between January 2006 and April 2007 from 123 survey sites where *T. repens* was present. At each site up to 50 stolons (approximately 5 cm long with 2-3 nodes and at least 1 m apart) were collected from a representative area which varied in size from 100 m2 to 10 ha, depending on plant density and habitat size). Stolons were transported on ice to CSIRO Black Mountain (S35 16 23.12 E149 06 49.27) and planted into 5 cm pots containing sterilised compost. Plants were kept covered with clear plastic in a growth room for two weeks, and then transferred to a climate controlled glasshouse maintained at an approximately 15/25°C night/day temperature regime for further growth.

2.2.3 *Virus detection*

Three methods were used to identify and quantify the presence of *Alfalfa mosaic virus* (AMV), *White clover mosaic virus* (WClMV) and *Clover yellow vein virus* (ClYVV) in *T. repens*: indicator-plant bioassays, polymerase chain reaction (PCR) and immunoassays. PCR was not used to detect WClMV and ClYVV as previous evidence indicated that WClMV and ClYVV could be reliably detected using bioassay (Godfree *et al.*, 2004b). A plant was declared virus-infected when at least two independent test results were positive for that virus. For all assays virus-positive sap controls were taken from *T. repens* plants collected near Canberra, ACT, Australia, while sap from plants grown from seed were used as negative controls. AMV, ClYVV and WClMV are not seed-transmitted in *T. repens* (Johnstone and Chu, 1993, Latch and Skipp, 1987). Indicator-plant bioassays, in which viral identification is based on symptoms in the leaves of the indicator plants cowpea (*Vigna unguiculata*) and *Chenopodium amaranticolor* were performed as described in Godfree *et al.* (2004b).

2.2.3.1 Detection of *Alfalfa mosaic virus* using RT-PCR and *Alfalfa mosaic virus*-specific PCR

Two sets of AMV specific PCR primers were utilised to detect AMV in *T. repens*. Initially AMV primers developed by Bariana *et al*. (1994) were used but the majority of the work was performed using primers F2 and R2 as described by Xu and Nie (2006) because it was found that the original primers had homology to a region of the AMV genome where variation was known to occur. *Trifolium repens* leaf tissue (<100 mg) was submerged in liquid nitrogen, ground quickly with a cold mortar and pestle and stored at -80°C. RNA was extracted using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol. Extracted RNA was stored in RNAse free water at -80°C.

Samples (5 µL, 100ng-5µg total RNA) of extracted RNA, 5.5 µL RNAse free H_2O and 2.0 µL (15-20 pmol) antisense primer were incubated for 5 min at 70°C in a Hybaid PCR Express (Integrated Sciences, NSW). Samples were held at 4°C while 4 µL MBI Fermentas 5x Reaction Buffer (RevertAid), 2 µL 5 mM dNTPs (final concentration of 1 mM) and 0.5 µL (10-20 units) RNAsin ribonuclease inhibitor (Promega) was added and incubated for 5 min at 37°C then held at 4°C while 1 µL (200 units) of M-MuLV Reverse Transcriptase (MBI Fermentas RevertAid) was added. Samples were then incubated for 60 min at 42°C, 10 min at 70°C and then incubated on ice if used immediately; otherwise samples were stored at -20°C.

Samples containing 5 µL of RT-PCR reaction, 5 µL 10x PCR Reaction buffer (Perkin Elmer), 3 µL 25 mM $MgCl_2$ (Applied Biosystems), 2 µL 5 mM dNTPs, 1 µL Forward primer (250 ng/µL), 1 µL Reverse primer (250 ng/µL), 0.5 µL (2.5 units) AmpliTaq DNA polymerase (Applied Biosystems) and RNAse free H_2O to a total volume of 50 µL were then treated according to the temperature regime described by Xu and Nie (2006). PCR products were visualised by separation on a 0.7% agarose gel run at 100 V for approximately 45 min with

GeneRulerTM 1 Kb Plus DNA Ladder as a standard (Fermentas). The gel was stained with ethidium bromide and bands were visualised under UV light.

2.2.3.2 Viral detection based on immunoassay

The immunoassay method used was based on the procedure described in Graddon and Randles (1986) with AMV antibodies provided by J. W. Randles (Adelaide University, South Australia). ClYVV and WClMV antibodies were provided by Paul W. G. Chu (CSIRO Plant Industry).

Trifolium repens leaf tissue (<0.5 g) was placed in a plastic bag with an equal volume (w/v) of phosphate buffered saline (PBS) (0.14 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) and crushed. The supernatant was transferred to a 1.5 mL Eppendorf tube and centrifuged at 10,000 g for 2 min. The supernatant was applied to nitrocellulose membrane in a series of 1 µl samples and air dried. The membrane was stored between filter paper at -20°C. The membrane was blocked by immersion in blocking buffer B containing PBS, 2.6% skim milk powder and 10% supernatant from healthy *T. repens* (2 g healthy leaf tissue crushed with an equal volume (w/v) of PBS and spun at 3000 rpm for 5 min) to absorb non-viral antibodies and was incubated for 15 min at 37°C with gentle shaking. The buffer was discarded, replaced with AMV, ClYVV or WClMV specific antibody diluted 1/1000 in blocking buffer B (PBS, 2.6% skim milk powder, 10% supernatant from healthy leaves) and incubated for 30 min at 37°C with shaking. The membrane was washed in Blocking buffer A (PBS, 2.6% skim milk powder) three times for three min. The buffer was discarded and the membrane immersed in alkaline phosphatase conjugated goat anti-rabbit gamma-globulin (Sigma chemicals) diluted 1/1000 in PBS containing 1% bovine serum albumin (BSA) and incubated for 30 min at 37°C with shaking. The nitrocellulose membrane was washed twice for three min in AP 7.5 (0.1 M Tris-HCl, 0.1 M NaCl, 2 mM MgCl2, 0.05% Triton X100, pH 7.5) and twice for three min in AP 9.5 (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM MgCl2, pH 9.5) at room temperature. The wash

was discarded and the substrate BCIP®/NBT-Blue Liquid Substrate System for Membranes (Sigma-Aldrich) added. The membrane was incubated at low light intensity with shaking until blue dots (positive samples) appeared. The substrate was drained and the Stop buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.5) added.

2.2.4 Statistical Analyses

2.2.4.1 Distribution of *Trifolium repens* and *Alfalfa mosaic virus*

Incidence of *T. repens* was compared across general habitat type (Table 2.1), conservation classification (very high, high, medium, low), disturbance class (high, medium, low) and proximity to cropping (≥ 1 km vs. < 1km) by Pearson's χ^2 goodness of fit test (Sokal and Rohlf, 1987). Contingency tables were created based on the number of sites with and without *T. repens* within each predictor variable group. One-way ANOVA was used to compare the mean abundance of white clover plants at each site across general habitat type, disturbance class and proximity to cropping categories. Post hoc means tests were undertaken using the Tukey-Kramer correction for multiple testing (Sokal and Rohlf, 1981).

All sites were examined to identify indicator species for the presence of *T. repens*. If species were observed at >5 sites then they were considered as possible indicators for the presence/absence of *T. repens*. To test for species-level associations between *T. repens* and different indicator species I tested for a departure from random expectation using $\chi 2$ contingency tests (Sokal and Rohlf, 1987).

The pattern of AMV frequency was compared across conservation, disturbance and crop proximity groups based on the numbers of sites with or without each virus (sites with T. repens only). Pearson's χ^2 was used to test goodness of fit (Sokal and Rohlf, 1987) unless >25% of expected cell counts were < 5, or if at least one cell had an expected count of <1, then Fisher's exact test (FET) was

used (Sokal and Rohlf, 1987). Generally, both χ^2 and FET provided similar results.

2.2.4.2 Single- and co-infection of *Trifolium repens*

Associations between AMV, ClYVV and WClMV were investigated based on 365 plants collected from 13 sites (ranging from 11 to 81 plants per site) from the study area. Only sites which had been tested for all three virus species were used for analysis. Initially the percentage of plants containing single (AMV+, ClYVV+, WClMV+), double (AMV+/ClYVV+, AMV+/WClMV+, WClMV+/ClYVV+) and triple (AMV+/ClYVV+/WClMV+) infections was determined. A test of association among virus species was then conducted using log linear analysis of the associated three-way (2 x 2 x 2) contingency table (Tabachnick and Fidell, 1996) containing the numbers of plants in each co-infection class. Saturated and unsaturated models (with no 3-way interaction) were compared using the likelihood ratio test statistic (G); model parameters were estimated by the maximum likelihood method. As the results of log linear analysis revealed the presence of a 3-way interaction term among the virus species, the presence of each pair of viruses split by presence or absence of the third virus was then tested for again using the likelihood ratio test statistic (G) as the test of association.

The same method was utilised to test for regional differences in association among the three viruses in plants collected from four sites in central NSW (n = 65 plants) and five sites in northern NSW (n = 131 plants) where all three viruses were detected. For the central NSW sites, log-linear analysis of the AMV x ClYVV x WClMV contingency table showed a significant 3-way interaction ($G_{(1)} = 6.9$, $P < 0.01$). Therefore the relationships between each virus pair was separately assessed in the presence and absence of the third virus. For the northern NSW sites a backward selection to choose the most parsimonious model was utilised, and a supplementary *G* test of association was made on the

AMV x ClYVV interaction with cell counts averaged over both WClMV classes (+/-).

All contingency analyses were undertaken in SAS Proc Genmod and Proc Freq version 9.1; GLM analyses were conducted using SAS Proc GLM version 9.1 (SAS Institute Inc., 2003).

2.3 Results

2.3.1 Distribution and abundance of Trifolium repens

Trifolium repens was found in 125 (59%) of the 213 sites surveyed in the study, (Figure 2.2 and Table 2.1). There was a distinct trend for *T. repens* to be most prevalent in cool, high altitude tableland and alpine regions of NSW, Vic. and the ACT (61-100% of sites) (Figure 2.2b), although it was also widespread along roadsides in the drier and warmer western slopes of NSW and in the Hunter Valley region. *Trifolium repens* populations were scattered or rare in coastal and low altitude areas (Figure 2.2b). Reflecting the geographic distribution, *T. repens* was less common in coastal and wetland habitats (≤ 25% of sites) than in inland grasslands, forests, woodlands, roadsides and other highly modified areas (≥ 75% of sites; $\chi^2_{(5)} = 65.5$, $P < 0.001$ across all habitat types; Figure 2.3a). *Trifolium repens* was present in an intermediate percentage of alpine sites (Figure 2.3a). Similarly, average *T. repens* abundance was highest in inland and modified habitats (average abundance score > 2, Figure 2.3c) and lowest in coastal and wetland habitats (Figure 2.3c) out of all sites surveyed. These differences were less obvious when only those sites containing *T. repens* where compared (i.e., abundance scores of ≥ 1; Figure 2.3d). When *T. repens* was present, coastal populations were less abundant than those found in modified and inland grassland habitats.

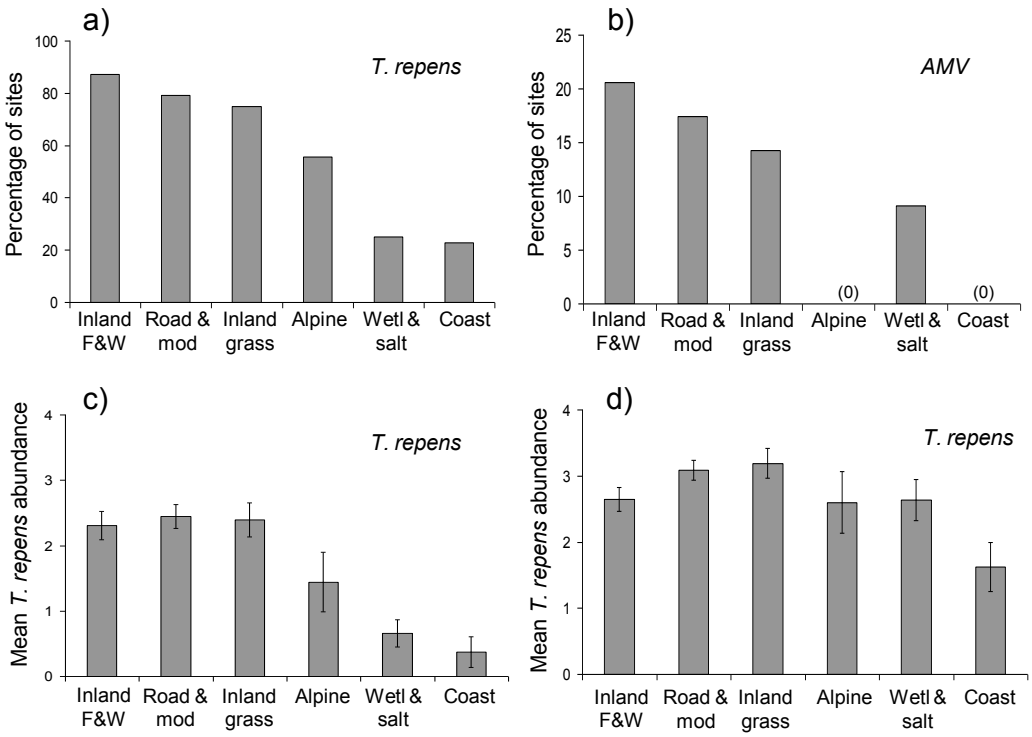


Figure 2.3: a) Percentage of surveyed sites containing *Trifolium repens* in each of the six main vegetation types; b) percentage of *T. repens* sites containing *Alfalfa mosaic virus* (AMV)*;* c) mean abundance of *T. repens* across all sites in each vegetation type; d) mean abundance of *T. repens* in sites that contained *T. repens* (i.e., excluding sites where *T. repens* was absent). For all figures: inland F&W = inland lowland, montane and subalpine forests and woodlands; Road & mod = Roadsides and modified vegetation on stock reserves; Inland grass = inland lowland, montane and subalpine grassland; Alpine = alpine bog, heath and snowpatch; Wetl & salt = wetlands, swamps and saltmarshes; Coast = coastal and coastal plain forests, woodlands and grasslands.

2.3.2 *Habitat affinity of Trifolium repens*

Of the 37 primary potential habitat types investigated in the study, *T. repens* was found in 27 (73%) (Table 2.1). These incorporated a wide range of native plant communities in addition to roadsides, revegetation sites and travelling stock reserves (TSRs). Most importantly, *T. repens* occurred at high site frequencies in a range of nationally or federally listed (Office of Legislative Drafting and Publishing, 2007, Environment ACT, 2005, Carter *et al.*, 2003) endangered native plant communities of very high conservation value, such as critically endangered white box-yellow box-Blakely's red gum grassy woodland and derived native grassland (7/7 sites) and endangered Natural Temperate Grassland of the Southern Tablelands of NSW and the ACT (17/20 sites). *Trifolium repens* was also prevalent in disturbed alpine areas within NPs (10/10 sites) and subalpine woodlands (15/18 sites). In contrast, *T. repens* was absent or rare in a range of potential habitat types, in particular the NSW endangered Brogo Wet Vine Forest, several Wetlands of National Significance (WNS), and a range of swamp or bog communities. *Trifolium repens* occurrence across SE Australia is summarised in Table 2.1 and further information regarding all the sites surveyed, including locations, can be obtained from the author.

Trifolium repens was non-randomly distributed across the conservation classes ($\chi^2_{(3)} = 26.1$, $P < 0.001$; Figure 2.4a), as low and moderate conservation-value sites had a greater rate of infestation (88% and 78% respectively) than high and very high conservation-value sites (41% and 51%). Mean abundance of *T. repens* at a site followed a comparable pattern (2.58 ± 0.27, 2.19 ± 0.26, 1.16 ± 0.26 and 1.40 ± 0.16 respectively for conservation categories: low, medium, high and very high respectively; $F_{3,209} = 7.8$, $P < 0.001$). Tthese differences were a result of the differences in the number of sites with *T. repens*, as average abundance did not change across those sites that contained *T. repens* (i.e. a minimum site abundance of 1 or more)($F_{3,121} = 0.1$, $P = 0.94$).

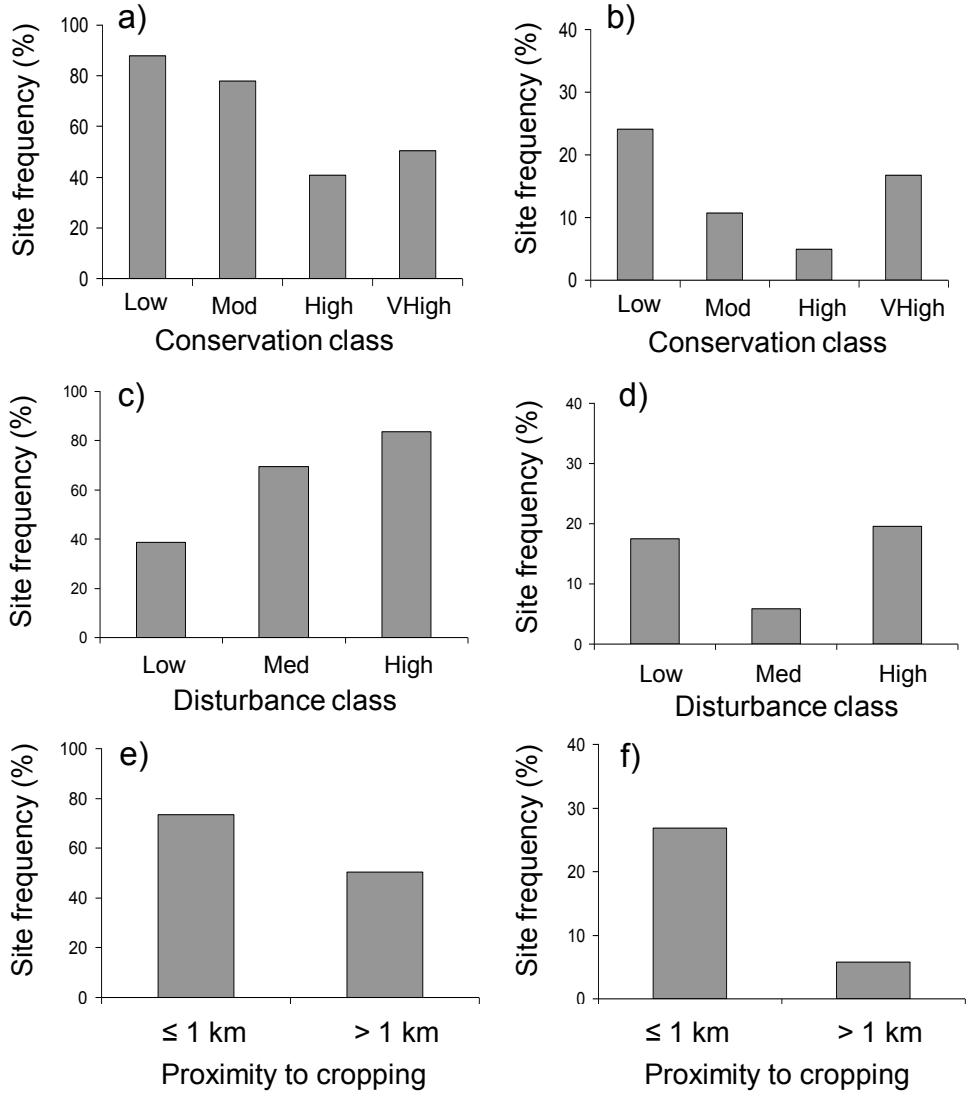


Figure 2.4: Factors influencing the presence of *Trifolium repens* and *Alfalfa mosaic virus* (AMV) in south-east Australia. a) Percentage of surveyed sites containing *T. repens* in each of the four conservation classes (low, moderate, high, very high); b) percentage of *T. repens* sites containing AMV in each conservation class; c) percentage of sites containing *T. repens* in each disturbance class (low, medium, high); d) percentage of *T. repens* sites containing AMV in each disturbance class; e) percentage of sites containing *T. repens* in relation to crop proximity; f) percentage of *T. repens* sites containing AMV in relation to crop proximity. All classification systems are described in the text.

The presence of *T. repens* was positively associated with the site disturbance classification ($\chi^2_{(2)} = 34.7$, $P < 0.001$), with 83% of highly disturbed sites containing *T. repens*, in contrast with 69% and 39% of sites with medium and low amounts of disturbance respectively (Figure 2.4c). Significant changes in abundance means ($F_{2,212} = 15.0$, $P < 0.001$) across disturbance classification (mean = 1.16 ± 0.15, 1.67 ± 0.22 and 2.51 ± 0.20 for low, medium and high respectively) demonstrated differences in infection frequency, although when only sites that contained *T. repens* were tested ($n = 125$). There was a weak trend ($F_{2, 122} = 3.6$, $P = 0.03$) for sites with medium disturbance to have a lower abundance (2.41 ± 0.18) than sites with low (2.98 ± 0.17) or high (3.00 ± 0.15) disturbance.

Trifolium repens was more likely to be present more frequently at sites within 1 km of cropped land (74% of sites) than in those >1 km away (50% of sites; $\chi^2_{(1)} = 11.0$, $P < 0.001$), and also had a higher average abundance of *T. repens* (1.97 ± 0.19 vs. 1.49 ± 0.14; $F_{1,211} = 4.4$, $P = 0.04$). The difference in means, which explained only 2% (model $R^2 = 0.02$) of variation in the data, was not significant when sites that contained *T. repens* were excluded from analysis ($F_{1,123} = 2.0$, $P = 0.16$).

2.3.3 *Indicator species for Trifolium repens*

The tendency for *T. repens* to occur in mesic and mid to high altitude environments, as k demonstrated above, was also reflected in the positive associations ($P < 0.05$) between presence of *T. repens* and indicator species including *Poa* spp. (mainly *P. labillardierei* and *P. costiniana*), *Eucalyptus pauciflora* and *Holcus lanatus*, which inhabit these types of environments. In contrast, *Banksia* spp., *Phragmites australis*, *Melaleuca* spp., *Glycine clandestina* and *Casuarina* spp. were all negatively associated with *T. repens*. The habitat affinities of each of these indicator species is provided in Table 2.2.

Table 2.2: Indicator plant species for *Trifolium repens* across the study area based on floristic data collected at 213 survey sites. Only species occurring in 10 or more sites are included. Species that have significant positive and negative associations with *T. repens* occur in >80% and ≤20% of sites containing *T. repens* respectively. Significance of association based on Fisher's exact test: ^{M}Marginal, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Species	Common habitat (National Herbarium of New South Wales, 1999-2009; Keith, 2004)	Number of sites where species was observed	Sites with *T. repens* (%)
Holcus lanatus	Widespread weed of mesic temperate areas	11	100**
Eucalyptus pauciflora	Alpine and subalpine (above 700 m in altitude) dry sclerophyll or grassy woodlands	33	87.9***
Mix of *Poa* spp.	Widespread, mainly mesic areas	13	84.6^{M}
Poa labillardierei	Mesic areas on river flats, open areas and forest	25	84**
Phragmites australis	Wet areas, particularly at the edge of bodies of water	10	20**
Casuarina spp.	Widespread	15	13.3***
Melaleuca spp.	Widespread	20	5***

2.3.4 *Prevalence of Alfalfa mosaic virus, White clover mosaic virus and Clover yellow vein virus in Trifolium repens populations*

AMV was detected at 19/125 (15%) of sites that contained *T. repens* (based on at least two methods of detection), and infected 4% to 91% (average 38%) of *T. repens* plants collected at these sites (Figure 2.2b and Table 2.1). No AMV was found in Victoria during the survey, and there was a strong tendency for AMV to infest white clover populations growing in northern NSW (Figure 2.2b). The presence of AMV was not associated with broad habitat type (P = 0.80, FET; Figure 2.4b), but eight of the sites containing AMV were in habitats listed as endangered or critically endangered, most notable being temperate grasslands of southern NSW and the ACT, and white box-yellow box-Blakely's red gum grassy woodlands and derived native grasslands (Figure 2.5).

There was no sign of a difference in the proportion of *T. repens* sites containing AMV among all four conservation value groups ($P = 0.30$, Fisher's exact test, FET; Figure 2.4b), or among disturbance classifications ($\chi^2_{(2)} = 3.2$, $P = 0.20$; Figure 2.4d). *Alfalfa mosaic virus* was more common in sites close to (≤1 km) cropping or agricultural white clover pastures ($\chi^2_{(1)} = 10.6$, $P < 0.01$) (Figure 2.4f), and was positively associated with *T. repens* abundance ($\chi^2_{(3)} = 8.0$, $P = 0.02$) across the three abundance groups (classes 1+2, 3 and 4). This indicated that AMV is more common at sites containing the most abundant *T. repens* populations [class 4 = 5/39 (13%) of sites; class 3 = 13/52 (25%) of sites in contrast with sites with low white clover abundance (combined classes one and two); 1/33 or 3% of sites].

All plants collected were tested for AMV, however, only plants from a subset of sites were also tested for ClYVV and WClMV (32 sites). ClYVV was detected in 30/32 sites (94%) at an average plant infection frequency of 36% (range 3-100%). WClMV was detected in 18/32 sites (56%) with an average infection frequency of 30% (range 1-89%) in infested

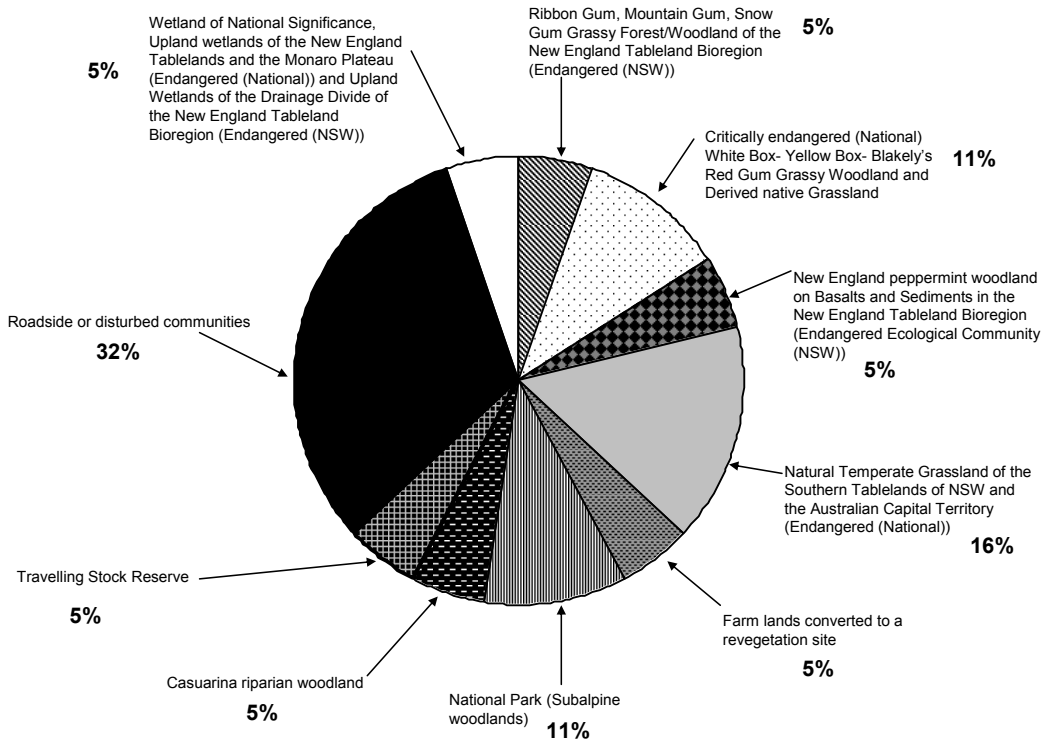


Figure 2.5: Percentage of sites containing *Alfalfa mosaic virus* (AMV) across all habitat types in which *T. repens* plants infected with AMV were collected. AMV was most frequently detected in roadsides/disturbed areas (32% of sites invaded by *T. repens* contained AMV) and temperate grassland (16%).

sites. Information about sites infested with AMV, WClMV or ClYVV is displayed in Table 2.1 and Appendix 2.2.

2.3.5 Viral infection patterns of Trifolium repens

Of the 365 clover plants taken from surveyed sites where all three viruses were detected, 74% were infected by one or more virus (47%, 41% and 19% were infected with AMV, ClYVV and WClMV respectively). Overall, 46% of plants were infected by one virus only (20% with AMV; 19% with ClYVV; and 7% with WClMV), 24% of plants were infected with two viruses (6% with AMV and WClMV; 17% AMV and ClYVV; and 1% ClYVV and WClMV) and 4% of plants were infected with all three viruses (Fig 2.6a). Of the plants infected with AMV, 42% were infected by AMV alone, and the remaining 58% were infected also by ClYVV, WClMV, or both viruses.

Significant differences were apparent in the regional prevalence of the three viruses (Figure 2.6b-c). AMV was much more common in northern NSW, occurring in 67% of plants, in contrast with central NSW (33%). WClMV was far less frequent in northern NSW compared with central NSW (18% vs. 30% of plants), whilst ClYVV was generally more common (31% vs. 21%) for these same regions. Co-infected plants were more frequent in central NSW than northern NSW (52% vs. 42% of infected plants), and 70% of AMV-infected plants were also infected with ClYVV, WClMV or both in central NSW, compared with 50% in northern NSW.

Tests of the distribution of AMV, ClYVV and WClMV infection across the 365 tested plants by Loglinear analysis demonstrated significant interactions between AMV, ClYVV and WClMV ($G_{(1)} = 6.00$, $P = 0.01$), with the association between any two given viruses dependent on the presence or absence of the third virus. Analysis of 2 x 2 contingency tables indicated that plants infected with both AMV and ClYVV appeared significantly ($G_{(1)} = 8.47$, $P < 0.01$) more

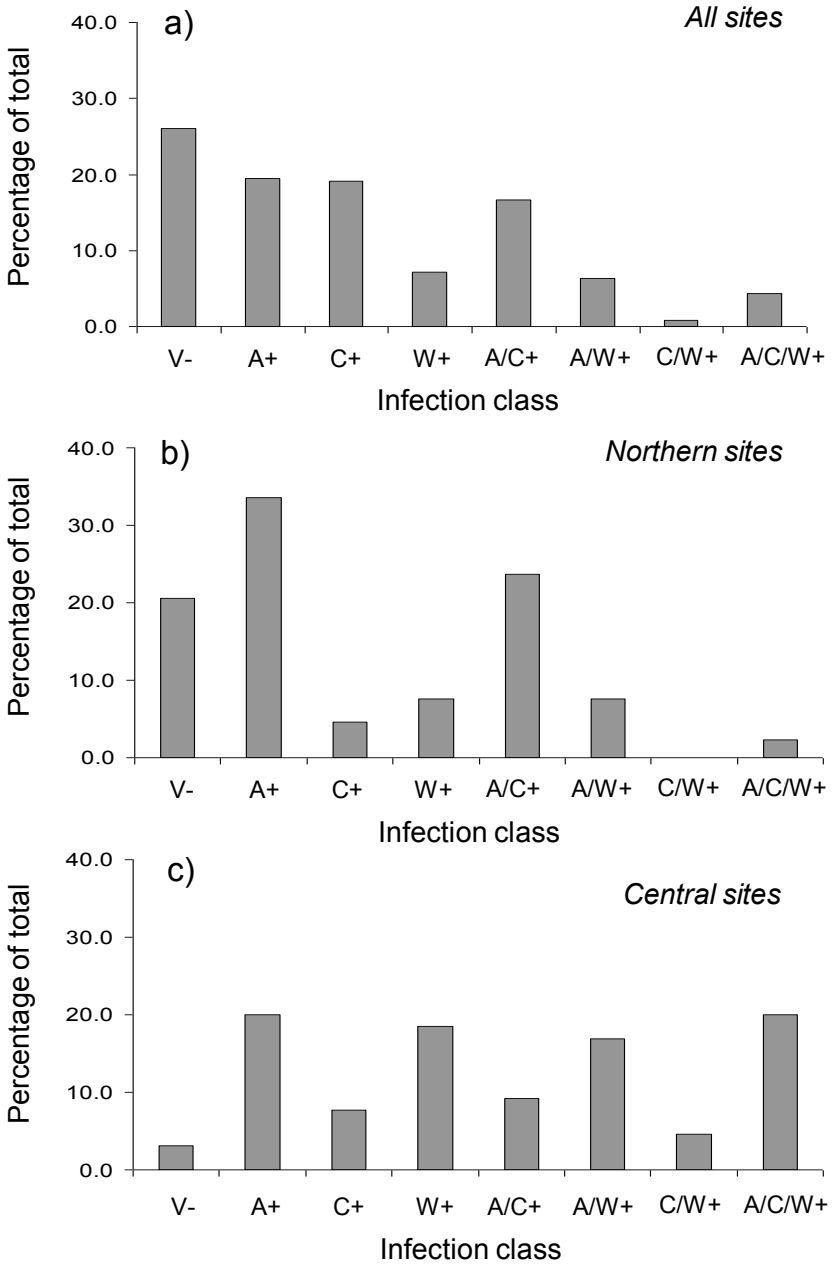


Figure 2.6: Co-infection frequencies among *Alfalfa mosaic virus* (AMV)*, Clover yellow vein virus* (ClYVV) and *White clover mosaic virus* (WClMV) across a) 315 *Trifolium repens* plants from 13 sites across New South Wales (NSW); b) 131 plants from 5 sites in northern NSW; and c) 65 plants from 4 sites in central NSW. For all bar graphs: V- = virus-free, A+ = AMV+, C+ = ClYVV+, W+ = WClMV+, A/C+ = AMV+ and ClYVV+, A/W+ = AMV+ and WClMV+, C/W+ = ClYVV+ and WClMV+, A/C/W+ = AMV+, ClYVV+ and WClMV+.

frequently than expected in the presence of WClMV (16/68 plants observed vs. 10.9/68 plants expected, equating to a 7.5% increase as a proportion of the total plant number), as did AMV and WClMV in the company of ClYVV (+4.1%, $G_{(1)} = 10.3$, $P < 0.01$). ClYVV and WClMV were generally negatively associated in the absence of AMV (-4.1% as a proportion of the total plant number, $G_{(1)} = 12.7$, $P < 0.001$). All other tests of two-way virus interactions were not significant ($P > 0.05$).

As the regional assessment of co-infection demonstrated that, in central NSW, the three-way interaction (AMV x ClYVV x WClMV) was significant ($G_{(1)} = 6.88$, $P < 0.01$), all two-way interactions were considered. AMV and ClYVV were positively associated in the company of WCMV ($G_{(1)} = 4.7$, $P < 0.05$), whilst AMV and WClMV, and ClYVV and WClMV were negatively associated in the absence of ClYVV and AMV respectively ($P < 0.05$ for both). In addition, in the northern sites, AMV and ClYVV were found to be positively associated ($G_{(1)} = 9.1$, $P < 0.01$), with co-infected plants approximately 5% more common (of the total number of plants sampled) than could be expected by chance.

2.4 Discussion

2.4.1 Distribution and abundance of Trifolium repens and associated viruses in south-eastern Australia

The aim of this study was to complete the first stages (Figure 2.1: stages 1 and 2) of the risk assessment of GM virus–resistant *T. repens* in south-eastern (SE) Australia, with a focus on determining the habitat-level potential for increased weediness of non-target populations following the release of transgenic and conventionally-bred *Clover yellow vein virus* (ClYVV)-, *White clover mosaic virus* (WClMV)- and especially *Alfalfa mosaic virus* (AMV)- resistant *T. repens* genotypes. The procedure involved identifying potentially at-risk non-target habitats within the study region to conduct a subsequent large-scale survey of *T. repens* and associated viruses (AMV, WClMV and ClYVV). This was the first such survey performed in Australia; while the prevalence of viruses in *T. repens*

populations in Australia has been well documented in agricultural settings (Norton and Johnstone, 1998), the range of native plant communities and other non-agricultural habitats containing this pathosystem remained largely unknown (Godfree *et al.*, 2004b).

Trifolium repens plants were detected at 59% of the sites visited in SE Australia, indicating that *T. repens* poses an ongoing invasion risk in many of the communities and habitats investigated. Furthermore, the survey was conducted between January 2006 and April 2007, during one of Australia's worst recorded droughts (Murphy, 2007), when most sites, especially in the southern part of the study region, were extremely dry. It is therefore likely that in more favourable seasons, *T. repens* prevalence would be higher. Nevertheless, my data show that *T. repens* inhabits a diverse range of plant communities ranging from low to very high conservation value, and despite favouring mesic disturbed areas, is not restricted to any particular habitat type. *Trifolium repens* occurs widely in mesic areas, river flats, woodlands, grasslands, and mid to high altitude alpine and subalpine dry sclerophyll or grassy woodlands (Table 2.1) across the entire 300,000 km^2 study region. These results indicate that to effectively complete the ecological risk assessment of transgenic virus-resistant *T. repens*, potential impacts need to be considered for numerous non-target community types across SE Australia, the implications of which are discussed below.

Besides being widely distributed, *T. repens* was abundant or very abundant at 67% of sites surveyed, with moderate and low levels of abundance observed at only 5% and 21% of sites respectively. Assuming that the impact of *T. repens* invasion on native plant communities is directly related to *T. repens* density, communities that may be at elevated risk of ecological damage include endangered temperate grasslands and grassy woodlands, alpine vegetation, and wet sclerophyll forests. The distribution and ecological characteristics of these communities, and the distribution of *T. repens* on the whole, indicate that

the array of native plant communities invaded by *T. repens* is at least partially currently limited by climatic conditions or a 'climatic envelope', a finding also supported by Hill (1996), and so it is likely that any shifts in climatic zones due to climate change (Thomas *et al.*, 2004) is likely to affect the regions and types of communities where *T. repens* is naturalised. Indeed, shifts in climatic zones could facilitate the invasion of *T. repens* into new areas, including endangered native plant communities. Consequently, any significant shift in the distribution of *T. repens* in the landscape is likely to necessitate reappraisal of the early stages of the risk assessment process.

A crucial element of the risk assessment process was to determine the distribution and abundance of AMV, WClMV and ClYVV within naturalised *T. repens*, and to investigate the factors which influence virus distribution in the landscape. My data show that of the sites that contained *T. repens*, 15% contained infestations of AMV, with 4-91% (mean = 38%) of plants infected at individual sites (Table 2.1 and Appendix 2.2). Six sites were roadsides or disturbed habitats, but the rest ranged from moderate to very high conservation value with eight sites listed as endangered or critically endangered (Figure 2.3). There was a clear geographical pattern of disease, with many of the sites containing AMV occurring in northern inland NSW (Figure 2.2a and b). The reason for the higher AMV prevalence in these areas is not known, but may be linked with recent rainfall patterns as, unlike southern NSW and Vic., these areas have not been afflicted with chronic (>8 year) drought (Murphy and Timbal, 2008).

When either WClMV or ClYVV were present, they had similar infection rates (average of 30% and 36% respectively) to AMV (an average of 38% of plants infected at a site). As AMV and ClYVV are both transmitted to *T. repens* by aphids in a non-persistent manner (Johnstone and Chu, 1993, Latch and Skipp, 1987), the similarity in observed infection rates is not surprising. Unlike ClYVV (and AMV), WClMV is dispersed by mechanical means and often occurs in

mown areas (Johnstone and Chu (1993). Although rates of infection were similar for all viruses, unlike AMV, ClYVV and WClMV exhibited no clear geographical pattern of disease distribution. Overall, AMV appears to be a less common virus across the landscape than WClMV (found at 56% of sites tested) and ClYVV (at 94% of sites tested). In addition, unlike AMV, ClYVV and WClMV were detected in Victoria, the Hunter region of NSW, and coastal NSW (Appendix 2.2). These large differences in virus distribution in the landscape particularly between AMV and ClYVV are difficult to explain, but could reflect the dynamics of aphid-borne viral transmission, differences in the specificity of aphid vectors (Wang *et al.*, 2006), transmission efficiency (Moreno *et al.*, 2005), variation in viral titre in host plants (Martín and Elena, 2009), or perhaps in the resistance of local *T. repens* genotypes to extant AMV genotypes (c.f., Godfree *et al.* 2009a). My data suggests that inferring viral distributions based on dispersal mechanisms for the purpose of risk assessment is unlikely to be reliable. It is also important to note that of the plants infected by AMV ≥12% were also infected with ClYVV, ≥4% with WClMV, and ≥2% with both ClYVV and WClMV. In addition, all regions in which AMV was found, except the Southern Tablelands, contained sites with both ClYVV and WClMV. Therefore the dynamics of viral co-infection may be important in this, and probably many other, multi-disease pathosystems.

Finally, a range of site-level factors were identified that related to AMV distribution in the landscape, including vegetation type, abundance of the host *T. repens*, and land management practice. Virus presence is likely to be associated with reservoirs of infection nearby and environmental conditions conducive to the development of large aphid populations or other dispersal mechanisms (Minks and Harrewijn, 1987), and may explain why AMV was more likely to be present in large *T. repens* populations. AMV may also be more likely to survive environmental or demographic variability when host densities are high, a well known phenomenon in H-P metapopulation dynamics. A

survey performed by Denny and Guy (2009) in New Zealand, indicated that AMV is more likely to occur in irrigated clover pastures (likely to have a high host densities). The landscape matrix is also clearly a crucial component in determining the presence/absence of AMV in naturalised *T. repens* populations, and in particular the presence of nearby potential sources of inoculum: I often observed that *T. repens* was more likely to be infected with AMV when lucerne (*Medicago sativa*), a key host of AMV, was growing within 1km of a given site. Indeed, in NSW there are at least 25 plant families that contain alternative host species for AMV (Appendix 2.3) (Hull, 1969, National Herbarium of New South Wales, 2009). Many of these hosts are introduced species that occur in agricultural landscapes, along roadsides or in disturbed plant communities, which may explain the tendency for AMV to occur in such habitats. Collectively these results indicate that future stages of the risk assessment process should focus on high conservation value habitats with a history of disturbance, large *T. repens* populations, and in close proximity to agricultural land containing hosts for AMV.

The use of "tiered risk assessment" is generally recommended for the risk assessment of transgenic plants (Wilkinson, 2003). The process starts with the first tier, by testing the "worst case scenario" under controlled conditions (i.e. lab or glasshouse). If results indicate that harm/exposure is negligible, then it can be concluded that risks are negligible. Following tier one tests, if there is concern regarding risk then tier two studies are undertaken. Tier two studies assess risk under more realistic conditions (i.e. field trials). If harm/exposure is not demonstrated to be negligible then tier three studies are undertaken (i.e. large scale-field trials) (Wilkinson and Tepfer, 2009). However, results obtained from tiered risk assessment alone (Figure 2.1: stage 3), without the habitat identification and large-scale field survey conducted beforehand (Figure 2.1: stages 1 and 2), would not have revealed the complex nature of this pathosystem.

2.4.2 *Implications for risk assessment of virus-resistant Trifolium repens in south-eastern Australia*

This study, which completes the first stages (Figure 2.1: stages 1 and 2) of the environmental risk assessment of virus-resistant *T. repens*, shows that *T. repens* is a common or abundant weed in a broad range of environments in SE Australia and that AMV is present in a minority (15%) of invaded sites. The *T. repens*-AMV pathosystem is most prevalent in northern NSW but is apparently absent in central and eastern Victoria (at least in the habitats studied). Other viruses (ClYVV and WClMV), which may be targeted in the future by transgenic pathogen-resistant (PR) *T. repens* genotypes, are more widely distributed (≥ 56% of sites tested) but associated host populations usually have similar overall infestation rates. As such, the movement of genes conferring resistance to any of these virus species from commercially grown *T. repens* genotypes to non-target host populations could potentially lead to increased weediness of *T. repens* in a wide range of threatened plant communities in SE Australia. To date, the magnitude of this effect has only been estimated for ClYVV in two plant communities (Godfree et al. 2009b).

AMV is a significant pathogen of *T. repens*, reducing growth by up to 33% (Latch and Skipp, 1987). At some sites AMV infestation was at a high frequency (91% of plants infected) where an impact on *T. repens* population dynamics therefore seems likely. Such frequencies are higher than those observed for ClYVV at any site in previous surveys (Godfree et al. 2004b). On the other hand, infection rates at some sites are sufficiently low (4%) that a major impact on *T. repens* populations is unlikely (although I cannot rule out the possibility that low AMV infection rates may reflect high virus-induced *T. repens* mortality under field conditions). Given that *T. repens* was abundant or very abundant at 18 of the 19 of sites infested with AMV (Appendix 2.2), any impact on naturalised *T. repens* populations is, therefore, likely to also result in an impact on the native plant community. The next stage of the risk assessment

process (Figure 2.1: stage 3) will focus on determining whether AMV resistance confers a fitness advantage to *T. repens* plants (the enemy-release hypothesis (Keane and Crawley, 2002), and whether the size of naturalised clover populations is being limited by AMV. If so, AMV-resistant *T. repens* would potentially pose a real risk to native plant communities in SE Australia. Similar studies would be necessary to resolve the level of risk associated with ClYVV- or WClMV- resistant genotypes.

2.4.3 General implications for risk assessment of disease-resistant plants

The results of this study have broader implications for the risk assessment of disease resistant plants that target single or multi-disease pathosystems. When gene pyramiding is employed to confer resistance to multiple pathogens the risk assessment must consider the distribution of all pathogens targeted, assumptions cannot be made regarding distribution, even if pathogens share a vector. I found that although AMV and ClYVV are dispersed similarly, their distribution in the landscape varied dramatically.

When considering resistance to a single virus, common co-infection, as in the case of *T. repens* by AMV, ClYVV and WClMV, may result in a reduction of the risks associated with the release of virus-specific resistant host genotypes. In relation to the effects of co-infection with different viruses Alves-Júnior *et al*. (2009) demonstrated that symptoms were equivalent in *Nicotiana benthamiana* plants co-infected with *Tomato rugose mosaic virus* (ToRMV) and *Tomato yellow spot virus* (ToYSV) to those generated by ToYSV alone. Therefore, any competitive advantage associated with resistance to one virus may be reduced as a result of compensatory effects of infection by other viruses. Consequently the potential risks associated with release of non-target white clover populations from the effects of an individual virus, i.e. AMV, ClYVV or WClMV, may be reduced. Risk assessments need to consider not only the effects of the specific virus, to which the plant is resistant, but also the possible compensatory effects of other pathogens present in the environment.

Finally, it is important to consider the relationship of non-target plant communities with agricultural lands where the disease resistant plant is likely to be grown. I found that those communities close to agricultural lands, with an abundant host population (often in regions where the host is grown commercially) or in disturbed areas (commonly due to agricultural activities) are more likely to be at risk following the release of AMV-resistant *T. repens*. If GM AMV-resistant *T. repens* is released commercially then these high risk sites are likely to be the ones closest to the site of commercial release and therefore the most difficult to protect. A detailed knowledge of H-P spatial distribution in the potential release area is a crucial component of any environmental risk assessment of disease resistant plants.

My results demonstrate the spatial and compositional complexity that can exist in widespread, natural multi-disease pathosystems, especially given that the factors influencing host-pathogen and pathogen-pathogen coexistence were numerous in this system. The process of a tiered risk assessment for PR species, in the absence of general principles that relate pathosystem structure, distribution and risk to non-target habitats, may lack rigour. While expensive and time-consuming to conduct, it is clear that if risk assessments of PR plants are to be effective they must account for variation in the target pathosystem.

3 Population structure and genetic diversity of *Alfalfa mosaic virus* from naturalised *Trifolium repens* in south-eastern Australia

3.1 Introduction

Previous research indicates that mechanisms which result in a reduction of the virus titre within a plant may result in the rapid selection of virus strains adapted to the new conditions (Roossinck, 1997). In many cases, the resistance in newly developed cultivars has been rapidly overcome by the adaptation of virus isolates (Fargette *et al.*, 2002, Harrison, 2002, García-Arenal and McDonald, 2003). Therefore, to stay abreast of viral evolution, plant breeders are continually seeking novel virus-resistant cultivars. Until recently control options available for the majority of viral plant diseases were limited (Bosch *et al.*, 2006), however, the development of transgenic virus-resistance in plants has added a new weapon to the plant breeder's arsenal.

In Australia, the Office of the Gene Technology Regulator (OGTR) must assess any potential risks a genetically modified (GM) plant may pose to the environment or to the health and safety of people before it can be released. Issues generally considered as part of a risk assessment of transgenic plants with virus resistance include: the risk of transgenes moving from the GM plant to an existing weed species; the extent to which transgenes can transfer from the GM crop to other non-GM crops; and of interest for this study, the potential for the targeted pathogenic organisms to become resistant to the technology.

The focus of this study is *Alfalfa mosaic virus* (AMV); a positive-sense single stranded RNA virus from the family *Bromoviridae*, genus *Alfamovirus*. The genome is tripartite with a fourth subgenomic coat protein (CP) mRNA (Hull, 2002). AMV is transmitted to white clover (*Trifolium repens* L.) by a number of aphid species in a non-persistent manner and glasshouse studies indicated that

AMV can reduce *T. repens* growth by up to 33% (Latch and Skipp, 1987, Johnstone and Chu, 1993). Symptoms may include a reduction in the number of leaves, stolons, rooting nodes, flower heads, seed production and nodulation as well as cold tolerance (Latch and Skipp, 1987). *Trifolium repens* is the most important pasture legume in many temperate regions of the world and is currently one of Australia's most abundant pasture crops (Bouton *et al.*, 2005). AMV infection of *T. repens* has been linked to major economic losses to the Australian dairy industry. In 1991, it was estimated that the annual loss in milk production in the Victorian dairy industry due to virus infection of clover exceeded A$20M (Garrett, 1991). To reduce yield losses resulting from AMV infection, GM AMV-resistant *T. repens* expressing the RNA 3 AMV CP gene has been developed for future commercialisation in south-eastern (SE) Australia (Spangenberg *et al.*, 2001). The CP sequence used for transgenic *T. repens* was sourced from an Australian AMV isolate. I will refer to the AMV CP sequence used for GM *T. repens* as the "GM_insert."

Since non-GM *T. repens* is already invasive in a wide range of high conservation-value plant communities in SE Australia (Chapter 2) and if resistance to AMV confers a fitness advantage to *T. repens*, then it may be hypothesised that GM AMV-resistant *T. repens* could pose an increased weediness threat to native plant communities in SE Australia due to the effect known as "enemy-release". The enemy release hypothesis proposes that non-indigenous species may become more successful invaders following reduced control by natural enemies (Colautti *et al.*, 2004) (see the research undertaken by Mitchell and Power (2003) for examples). Therefore, it is postulated that transgenic *T. repens* could pose a further significant risk to native ecosystems in this SE Australia. For this reason an environmental risk assessment of transgenic *T. repens* is crucial before any commercial release.

An important component of this assessment involves determining the population structure and genetic diversity of AMV infecting naturalised

T. repens in SE Australia. Genetic diversity can be defined as the likelihood that two randomly selected individuals from a population are different (García-Arenal *et al.*, 2001). Furthermore, genetic diversity is a product of the number of variants (unique isolates of the same virus) in a population, the frequency of each variant present in the population and the genetic distance among variants (García-Arenal *et al.*, 2001). Evolutionary potential, as a function of the amount of genetic diversity (Duffy *et al.*, 2008), recombination, gene flow and population size, is likely to be strongly related to the durability of resistance (García-Arenal and McDonald, 2003). Previous research indicates that recombination increases the number of variants in a population (Ramos-Onsins and Rozas, 2002). Virus species that display more genetic plasticity appear more likely to overcome resistance than virus species where recombination or reassortment is rare. Additionally, a high degree of gene flow increases the likelihood that resistance genes present in the pathogen population will come in contact with the resistant plants (García-Arenal and McDonald, 2003). It is important to establish whether virus resistance, conferred by the expression of the GM_insert, may provide resistance to AMV in this study region, or whether sufficient AMV CP gene diversity and therefore potential for AMV to overcome resistance, already exists in SE Australia.

To date, previous sequence analysis of the AMV CP gene has been predominantly based on host type and the characterisation of nucleotide sequence variability (Parella *et al.*, 2000, Xu and Nie, 2006, Mih and Hanson, 1998). However, no information is currently available regarding the population structure or genetic diversity of SE Australian AMV, it is therefore unknown how successful the GM_insert would be at providing resistance for *T. repens* against Australian variants if commercial release was approved. Partial virus resistance in GM peas containing AMV CP sequence has been demonstrated (experimental conditions only) (Timmerman-Vaughan *et al.*, 2001). Varying

degrees of AMV resistance in GM barrel medic and GM burley tobacco has also been demonstrated (Jayasena *et al.*, 2001, Xu *et al.*, 1998).

Numerous virus resistance genes in plants have been overcome by viruses with four or less amino acid changes (Harrison, 2002). Taschner *et al*. (1994) demonstrated that a CP transgene sourced from wild-type AMV provided resistance for transgenic tobacco against an AMV mutant, with only one amino acid change. However, a CP transgene sourced from an AMV mutant, with only one amino acid change, was unable to provide resistance for transgenic tobacco against wild-type AMV. Indeed, amino acid similarity between the CP amino acid sequence of Australian AMV and the amino acid sequence of the GM_insert used for transgenic *T. repens* is likely to be vital for the longevity of resistance. If high genetic diversity is present within the SE Australian AMV population there is likely to be potential for the disease to overcome resistance. In addition, commercial release of a transgenic organism is likely to result in an alteration of the host dynamics. This, in turn, may change the pathosystem to increase selection pressures on AMV, which is already reported as widespread in agricultural systems in Australia (Norton and Johnstone, 1998, Jones, 2004b, McKirdy and Jones, 1997), and potentially result in the emergence of novel virus species and strains (Fargette *et al.*, 2006). Acosta-Leal *et al.* (2010) found that tomato virus diversity increased significantly when the strength of host resistance increased.

Although viruses have small genomes, they are easy to culture/ maintain, they appear to have more rapid rates of evolution than eukaryotic species and they have short generation times (Duffy *et al.*, 2008), little work has been undertaken to understand RNA virus population genetics (Moya *et al.*, 1993). Furthermore few studies have analysed RNA virus populations in natural (rather than agricultural) systems (Seabloom *et al.*, 2009a, Seabloom *et al.*, 2009b). Given phylogenetic methods are generally not used when studying new virus sequences (Duffy and Seah, 2010), the addition of population genetics to

studies would appear justified. As AMV infects *T. repens* that is invasive in a diverse range of environments in SE Australia, my study focused also on understanding diversity and distribution of the virus within the context of the broader landscape matrix.

The key objective of my work is assessing the evolutionary potential of AMV in the SE Australian region to overcome CP-conferred resistance in GM *T. repens.* Specifically I generate and analyse the RNA 3 CP sequence of AMV isolates from naturalised SE Australian *T. repens* populations from a variety of habitat types in order to determine the genetic diversity, population structure, amount of gene flow (i.e. the spatial distribution of variants), selection, recombination and the likely source regions or countries of SE Australian AMV. Knowledge of the source of Australian AMV could provide a connection with studies of AMV populations undertaken elsewhere and other transgenic plants with AMV CP-mediated resistance. Additionally I assess if there is a relationship between the genetic diversity of AMV and i) vegetation type; ii) geographic location; iii) conservation value of the habitat; and iv) the amount of disturbance at the collection site.

3.2 Methods and Materials

3.2.1 Alfalfa mosaic virus collections from Trifolium repens

Trifolium repens plants infected with AMV were obtained from a survey of naturalised *T. repens* populations from a wide range of habitats in SE Australia as described in Chapter 2. Plants were randomly selected, even if symptoms were not apparent and tested for AMV (see methods and results in Chapter 2). For sequence analyses AMV infected *T. repens* plants were sampled from 19 different sites in NSW. To gain an understanding of within site diversity two sites, *Coolah2* and *Carinya*, were sampled extensively (18 and 31 sequences, respectively). These two sites were chosen because they were from different regions (separated geographically) and both had a high frequency of plants infected, 95% of plants tested from *Coolah2* were infected with AMV and 94%

from *Carinya*. To study between-site diversity up to six sequences were gained from each of the remaining 17 sites (Table 3.1). Independent samples were collected and sequenced more than once from some AMV infected plants, if different variants were found from the same host plant, these were identified by S1, S2, S3 etc in the sequence name. A total of 83 sequences were used for population analysis.

3.2.2 Sample preparation

Fresh *T. repens* leaf tissue (<100 mg) was submerged in liquid nitrogen, ground with a cold mortar and pestle and stored at -80oC. RNA was extracted using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol. Extracted RNA was stored in RNAse free water at -80oC.

3.2.3 Sequencing the Alfalfa mosaic virus RNA 3 coat protein gene

3.2.3.1 Reverse Transcriptase-PCR (RT-PCR)

Samples (5 μL, 100ng-5μg total RNA) of extracted RNA, 5.5 μL RNAse free H_2O and 2.0 μL (15-20 pmol) antisense primer AMV-R2 (Xu and Nie, 2006) were incubated for 5 min at 70°C in a Hybaid PCR Express (Integrated Sciences, NSW). Samples were held at 4°C while 4 μL MBI Fermentas 5x Reaction Buffer (RevertAid), 2 μL 5 mM dNTPs (final concentration of 1 mM) and 0.5 μL (10-20 units) RNAsin ribonuclease inhibitor (Promega) was added and incubated 5 min at 37°C then held at 4°C while 1 μL (200 units) of M-MuLV Reverse Transcriptase (MBI Fermentas RevertAid) was added. Samples were then incubated for 60 min at 42°C, 10 min at 70°C and then incubated on ice if used immediately; otherwise samples were stored at -20°C.

3.2.3.2 *Alfalfa mosaic virus* specific PCR

Samples containing 5 μL of RT-PCR reaction, 5 μL 10x PCR Reaction buffer (Perkin Elmer), 3 μL 25 mM $MgCl_2$ (Applied Biosystems), 2 μL 5 mM dNTPs, 0.5 μL (2.5 units) AmpliTaq DNA polymerase (Applied Biosystems), 1 μL Forward primer (250 ng/μL) and 1 μL Reverse primer (250 ng/μL), AMV-F2 and AMV-R2 respectively (Xu and Nie, 2006), and RNAse free H_2O to a total

Table 3.1: Analysis of sequence polymorphism of AMV CP obtained from infected naturalised *T. repens*. Sequences were grouped by collection site for analysis.

Site	Region[a]	Conservation value[b]	Disturbance class[b]	Community type[c]	Abundance of *T. repens* at site[d]	AMV frequency (%)[e]	No. sequences	No. variants	Total no. nucleotides[f]	Polymorphic sites	Pi[g]	Pi (variants only)
All sites	-	-	-	-	-	-	83	58	573	49	0.00914	0.01103
Hon	ST	L	H	1	3	Unknown	1	1	580	-	-	-
Pep	ST	VH	L	2	4	11.1	6	3	579	7	0.00403	0.01103
Canob1	CT	M	M	3	3	47.4	1	1	580	-	-	-
Canob2	CT	H	L	3	1	9.1	2	2	580	7	0.01207	0.01207
Coolah1	CT	L	H	1	3	60.0	1	1	580	-	-	-
Coolah2	CT	L	H	1	3	76.2	18	8	580	19	0.00637	0.01151
Orange	CT	L	H	1	3	16.7	2	2	579	9	0.01554	0.01554
Carinya	NWSP	M	H	1	3	90.6	31	21	579	38	0.01291	0.01498
Castle	NWSP	M	H	4	3	79.0	4	4	580	15	0.0136	0.0136
Cumnock	NWSP	L	H	1	3	77.3	3	3	580	14	0.01609	0.01609
Nund	NWSP	VH	L	5	3	3.5	1	1	580	-	-	-
GI3#2 & GI4*	NT	VH	L	6 & 7 respectively	4	23.7	2	2	580	7	0.01207	0.01207
INR	NT	L	H	1	Unknown	Unknown	4	4	580	11	0.00977	0.00977
INV	NT	VH	M	4	3	11.8	1	1	580	-	-	-
Llan	NT	VH	L	8	4	25.0	1	1	580	-	-	-
Terry	NT	L	H	1	3	81.8	2	1	580	0	0	-
Bren	SCI	L	H	9	3	6.7	2	2	580	2	0.00345	0.00345
Stoney	ST	VH	L	10	3	3.5	1	1	580	-	-	-

*Sites GI3#2 and GI4 were combined because each site had only 1 isolate and the sites were separated by <10km.

[a]Geographic regions include Central Tablelands (CT), North West Slopes and Plains (NWSP), Northern Tablelands (NT), Southern Tablelands (ST) and South Coast and Illawarra (SCI) NSW.

[b]L = low, M = medium, H = high and VH = very high.

[c]1= Roadsides and modified vegetation, 2 = Subalpine sod-tussock grassland, 3 = Subalpine woodland, 4 = White Box- Yellow Box- Blakely's Red Gum Grassy Woodland and Derived native Grassland, 5 = Casuarina woodland, 6 = New England peppermint woodland on Basalts and Sediments in the New England Tableland Bioregion, 7 = Ribbon Gum, Mountain Gum, Snow Gum Grassy Forest/Woodland of the New England Tableland Bioregion, 8 = Upland wetlands of the New England Tablelands and the Monaro Plateau, Upland Wetlands of the Drainage Divide of the New England Tableland Bioregion, 9 = Travelling stock reserves, 10 = Natural Temperate Grassland of the Southern Tablelands of NSW and the Australian Capital Territory.

[d]Abundance of *T. repens* at sites from a previous survey (Chapter 2).

[e]AMV frequency = % *T. repens* plants infected that were collected from the site.

[f]The total number of nucleotides used for analysis (excluding sites with missing data or ambiguities).

[g]Nucleotide diversity.

volume of 50 μL were treated according to the temperature regime described by Xu and Nie (2006). PCR products were visualised on a 0.7 % agarose gel stained with ethidium bromide under UV light. GeneRuler™ 1 Kb Plus DNA Ladder (Fermentas) was used as a standard. PCR cleanup was conducted with Millipore 96 well clean-up plates (MANU03010) according to the manufacturer's protocol.

Forward and reverse reactions were set up in a MicroAmp® 96-well reaction plate with primers AMV-F2 and AMV-R2 respectively. Reactions contained 2 μL ABI BigDye® Terminator v3.1, 3 μL BigDye® Sequencing Buffer, 2 μL primer (AMV-F2 or AMV-R2), and 13 μL cleaned PCR product. The plate was then incubated in a Hybaid PCR Express (Integrated Sciences, NSW) (ramp speed 1) for 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C, for 25 cycles then held at 4°C.

DNA samples were precipitated by adding 2 μL 3 M sodium acetate (pH 5.2), 2 μL 125 mM EDTA (pH 8) and 50 μL 99-100% ethanol. Samples were incubated at room temperature in the dark for 15 min, centrifuged 30 min at 3000 g at 4°C, inverted and spun to remove excess liquid. Ethanol (100 μL 70% ethanol) was added to each sample and centrifuged for 10 min at 1600 g at 4°C. The plate was inverted to remove liquid, another 100 μL 70% ethanol was added to samples, which were centrifuged for 10 min at 1600 g at 4°C. The plate was inverted to remove excess liquid, then vacuum centrifuged (John Morris Scientific Pty Limited) for approximately 30 min. DNA pellets were sent to the ACRF Biomolecular Resource Facility (The John Curtin School of Medical Research, The Australian National University, Canberra, Australia) for sequencing on an AB 3730 capillary Sequencer (Applied Biosystems).

3.2.4 Sequence analyses

3.2.4.1 *Alfalfa mosaic virus* genetic diversity

Sequences were edited and aligned using Sequencher 4.8 (Gene Codes Corporation, 2007). All sequences were trimmed to the same length (580

nucleotides). Sequence homology was calculated in BioEdit (Hall, 1999). Sequences were grouped by collection site (Table 3.1), community type (Table 3.2), geographic region (Table 3.3), conservation value (Table 3.4) and disturbance level (Table 3.5) for analyses. Criteria for the classification of sites into conservation value and disturbance level groups are contained in the methods section of Chapter 2. Number of polymorphic sites, number of variants (unique sequences), nucleotide diversity and the ratio of the rate of non-synonymous substitutions to the rate of synonymous substitutions (ω ratio) were calculated in DnaSP (Rozas *et al.*, 2003). Amino acid diversity was detected using MEGA software (Tamura *et al.*, 2007).

An analysis of molecular variance (AMOVA) was conducted in GenALEx (Peakall and Smouse, 2006) to estimate the relative contributions of local sites, regional geographic separations and habitat types on overall AMV genetic distribution. For AMOVA analysis (99 permutations) any sites with only one sequence were excluded from the analysis (56 sequences remained). Sequences were grouped by site, region, community type, conservation value, or disturbance level for analyses.

Phylogenetic relationships between the Australian AMV CP sequences were inferred by the tree producing software SplitsTree4 (Huson and Bryant, 2006). Rooted (rooted with a randomly chosen sequence) and unrooted nucleotide neighbour joining (NJ) phylograms, based on aligned nucleotide sequences, were produced with bootstrap values (1000 replications) on branches.

3.2.4.2 Recombination

The quantification of recombination can provide insight into the evolutionary potential of a population, but if recombination is present within a population patterns of common ancestry can be confused as sequences with separate evolutionary histories have their genomes joined (Duffy and Seah, 2010). If recombination has occurred between virus isolates, then a phylogenetic tree may not correctly represent the relatedness of the isolates. Tests for

Table 3.2: Analysis of AMV CP sequence polymorphism. Sequences were grouped by native plant community type for analysis.

Community type	Sites	No. sequences	No. variants	Total no. nucleotides[a]	Polymorphic sites	Pi[b]
Roadsides, modified vegetation and stock reserves	Cumnock, Terry, INR, Orange, Coolah1, Coolah2, Hon, Carinya and Bren	64	43	577	47	0.01031
Inland lowland montane and subalpine forests and woodlands	Nund, Castle, Canob1, Canob2, INV, GI3#2 and GI4	11	11	575	25	0.0117
Lowland montane and subalpine grasslands	Stoney and Pep	7	4	578	11	0.00709
Wetlands, swamps and saltmarshes	Llan	1	1	580	-	-

[a]The total number of nucleotides used for analysis (excluding sites with missing data or ambiguities).
[b]Nucleotide diversity.

Table 3.3: Analysis of AMV CP sequence polymorphism. Sequences were grouped by geographic region for analysis.

Region	Sites	No. sequences	No. variants	Total no. nucleotides[a]	Polymorphic sites	Pi[b]
Central Tablelands	Canob1, Canob2, Coolah1, Coolah2 and Orange	24	13	579	28	0.00812
North West Slopes and Plains	Carinya, Castle, Cumnock and Nund	39	29	575	45	0.01212
Northern Tablelands	GI3#2 & GI4*, INR, INV, Llan and Terry	10	9	580	17	0.00966
South Coast and Illawarra	Bren	2	2	580	2	0.00345
Southern Tablelands	Hon, Stoney and Pep	8	5	579	13	0.00785

[a]The total number of nucleotides used for analysis (excluding sites with missing data or ambiguities).
[b]Nucleotide diversity.

Table 3.4: Analysis of AMV CP sequence polymorphism. Sequences were grouped by conservation value for analysis.

Conservation value	Sites	No. sequences	No. variants	Total no. nucleotides[a]	Polymorphic sites	Pi[b]
Low	Coolah1, Coolah2, Orange, INR, Terry, Cumnock, Bren and Hon	33	22	578	30	0.00825
Medium	Canob1, Carinya and Castle	36	26	574	40	0.01079
High	Canob2	2	2	580	7	0.01207
Very high	GI3#2, GI4, INV, Llan, Nund, Pep and Stoney	12	9	578	17	0.00957

[a]The total number of nucleotides used for analysis (excluding sites with missing data or ambiguities).
[b]Nucleotide diversity.

Table 3.5: Analysis of AMV CP sequence polymorphism. Sequences were grouped by disturbance class for analysis.

Disturbance class	Site	No. sequences	No. variants	Total no. nucleotides[a]	Polymorphic sites	Pi[b]
Low	Canob2, GI3#2, GI4, Llan, Nund, Pep and Stoney	13	10	578	19	0.01003
Medium	Canob1 and INV	2	2	579	10	0.01727
High	Coolah1, Coolah2 Orange, INR, Terry, Carinya, Castle, Cumnock, Bren and Hon	68	47	573	46	0.00885

[a]The total number of nucleotides used for analysis (excluding sites with missing data or ambiguities).
[b]Nucleotide diversity.

recombination between the AMV CP sequences collected were performed in SplitsTree4 (Huson and Bryant, 2006), DnaSP (Rozas *et al.*, 2003), RAT (Etherington *et al.*, 2005), Recom58 from SNAP Workbench (Griffiths and Marjoram, 1996), GENECONV (Sawyer, 1989) and Recco (Maydt and Lengauer, 2006). Numerous programs were used because there are a number of methods to detect recombination (Posada *et al.*, 2002), and many programs have limitations (Etherington *et al.*, 2005).

3.2.4.3 Selection

Selection can influence the genetic structure of a virus population (García-Arenal *et al.*, 2001), therefore statistical tests of neutrality, Tajima's D (Tajima, 1989) and Fu and Li's F and D statistic (Fu and Li, 1993), to detect the influence of natural selection on the SE Australian AMV population, were performed in DnaSP (Rozas *et al.*, 2003). The purpose of these tests was to determine if the evolution the SE Australian AMV population is occurring randomly ("neutrally") or non-randomly (the population is undergoing selection).

3.2.4.4 Spatial genetic structure and host association

A haplotype network was employed to test whether AMV infecting naturalised *T. repens* in SE Australia exhibits geographic structure by site. The haplotype network generated in SplitsTree4 (Huson and Bryant, 2006) was composed of Australian AMV CP sequences and the CP used for transgenic white clover (GM_insert). Bootstrap values (1000 replications) were calculated in SplitsTree4 (Huson and Bryant, 2006).

Principal coordinates analysis (PCA) was conducted to test for clustering of populations by site, region or habitat type. Sequences were grouped by site for PCA analysis in GenALEx (Peakall and Smouse, 2006). Isolation by distance analysis (Mantel test) using GenALEx (Peakall and Smouse, 2006) was undertaken to test for any correlation between geographic and genetic distance of AMV in SE Australia. The number of migrants (Nm) was also estimated in

GenALEx (Peakall and Smouse, 2006) to determine the amount of geneflow between sites.

In order to test for relationships between AMV and host and/or geographic origin, available AMV CP nucleotide sequences were obtained by a nucleotide BLAST search (National Center for Biotechnology Information (NCBI), 2005) and aligned using ClustalW2 (Higgins *et al.*, 1994). Rooted nucleotide NJ phylograms (rooted with a randomly chosen sequence) containing Australian and international sequences were created with bootstrap values (1000 replications) on branches in SplitsTree4 (Huson and Bryant, 2006).

3.2.4.5 Comparison of Australian *Alfalfa mosaic virus* sequences to the coat protein insert used for transgenic *Trifolium repens*

The CP insert used for GM *T. repens* (GM_insert) provided by Dr. Bill Taylor (CSIRO Plant Industry Business Development Advisor) was aligned with the collected Australian sequences using BioEdit version 7.0.9.0 (Hall, 1999). Phylogenetic relationships between the collected CP sequences and the GM_insert were inferred by the tree producing software SplitsTree4 (Huson and Bryant, 2006). Rooted (rooted with a randomly chosen sequence) and unrooted nucleotide NJ phylograms based on aligned nucleotide sequences were produced. Bootstrap values were calculated in SplitsTree4 (Huson and Bryant, 2006) with 1000 replicates. Percentage identity/similarity was calculated between Australian AMV nucleotide and the GM_insert using BioEdit version 7.0.9.0 (Hall, 1999) to predict the ability of the GM-insert to confer resistance to the AMV population in SE Australia.

3.2.4.6 Predicted *Alfalfa mosaic virus* secondary protein structure

All sequences were translated into amino acid sequences and percentage (%) homology between sequences was determined using sing BioEdit version 7.0.9.0 (Hall, 1999). As amino acid changes can impact on protein folding and potentially result in reduced recognition of the virus by transgenic *T. repens*,

secondary protein structure of the AMV CP amino acid sequences was predicted using SWISS-MODEL (Arnold *et al.*, 2006).

3.3 Results

3.3.1 Alfalfa mosaic virus genetic diversity

RNA 3 was successfully sequenced from 83 AMV infected *T. repens* plants collected from 19 different sites in NSW (Figure 3.1 and Table 3.1). When sequences were aligned and trimmed to 580 bases (Appendix 3.1), 58 variants were identified (the number of unique sequences in the population), with 49 polymorphic sites resulting in a low total nucleotide diversity (Pi) of 0.009 (Table 3.1), amino acid diversity of 0.005 and ω ratio of 0.075. No indels (insertions or deletions) were observed when comparing the Australian CP AMV nucleotide sequences in an alignment (Appendix 3.1). To prevent overrepresentation of clones in the dataset for allele based analyses, only the variants observed (unique sequences in a group) were subject to further analyses.

Nucleotide diversity of AMV at individual sites ranged from 0.00303 to 0.0136. When sequences were grouped by community type (Table 3.2) or conservation value (Table 3.4) nucleotide diversity was similar across community types/classes. However, when sequences were grouped by region (Table 3.3) sequences from the South Coast and Illawarra region (from site *Bren*) had a lower Pi (0.00345) than the other region groups. When sequences were grouped by disturbance level (Table 3.5), the group of sequences with high disturbance (from site *Orange*, *INR*, *Terry*, *Carinya*, *Castle*, *Cumnock*, *Bren* and

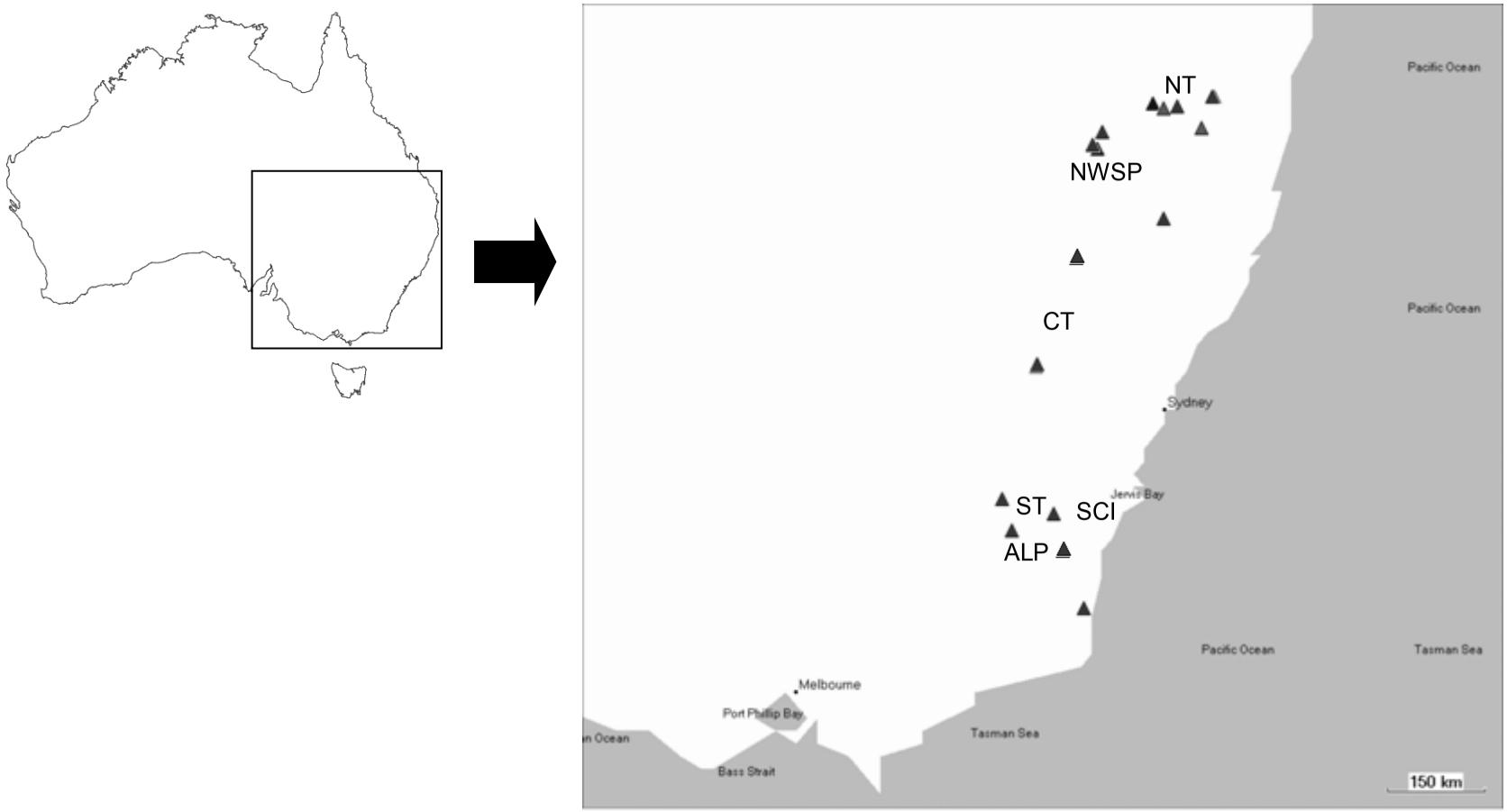


Figure 3.1: Location of sites where AMV was collected from naturalised *Trifolium repens* populations. Triangles indicate the location of sites with AMV. Geographic regions containing AMV infected included Alpine (ALP), Central Tablelands (CT), North West Slopes and Plains (NWSP), Northern Tablelands (NT), Southern Tablelands (ST) and South Coast and Illawarra (SCI) NSW.

Hon) had the lowest Pi (0.00885) which was half the Pi value for the group of sites with medium disturbance (0.01727).

Nucleotide sequence identity/similarity of all AMV sampled ranged from 100% to 97.2% (nucleotide sequence identity/similarity data not shown). There were 14 groups of sequences identified that had 100% nucleotide sequence similarity. Nine of these groups contained AMV sequences that originated from different sites. The largest group (Group 14) contained 22 (26.5% of the total population) identical sequences from eight different sites. Additionally, there were 26 (31.3% of the sequences) unique CP sequences (not found at any other site) (Table 3.6).

When sequences grouped by sites and regions were analysed by AMOVA, 0% of the sequence variation was partitioned within regions, 17% among sites and 83% within sites (Table 3.6a). The number of migrants (Nm) was 4.6. Statistics from the AMOVA indicate that the correlation within the regions, relative to the total (PhiRT) was 0.09 (*P* = 1.000*)*; correlation between sequences within sites, relative to sequences from the same region (PhiPR) was 0.14 (*P* = 0.010*)*; and the correlation between sequences within sites relative to the total (PhiPT) was 0.05 (*P* = 0.120*)*. Similar results were obtained when sites were grouped by conservation value: For example, 0% of the variation was explained by conservation value (Table 3.6c), PhiRT was -0.071 (*P* = 0.96), PhiPR was 0.155 (*P* = 0.02) and PhiPT was 0.096 (*P* = 0.01) (Table 3.7c); Nm = 4.7. When sites were grouped by community type, 6% of the molecular variance occurred among community types, 8% among sites and 86% within sites (Table 3.7b), with PhiRT = 0.057 (*P* = 0.06), PhiPR = 0.086 (*P* = 0.03) and PhiPT = 0.139 (*P* = 0.03); Nm = 3.1. When classified by disturbance level, 1% of molecular variance was among disturbance classes, 3% among sites and 96% within sites (Table 3.7d),

Table 3.6: Summary AMOVA tables (see Tables 3.2-5 for classification groups).

a) Molecular variance of sites classified by region.

Source	df	SS	MS	Est. Var.	%
Among regions	4	19.914	4.978	0.000	0%
Among sites	6	33.890	5.648	0.630	17%
Within Sites	66	206.625	3.131	3.131	83%
Total	76	260.429		3.761	100%

b) Molecular variance of sites classified by conservation value.

Source	df	SS	MS	Est. Var.	%
Among conservation value class	3	14.225	4.742	0.000	0%
Among sites	7	39.578	5.654	0.576	16%
Within sites	66	206.625	3.131	3.131	84%
Total	76	260.429		3.707	100%

c) Molecular variance of sites classified by community type.

Source	df	SS	MS	Est. Var.	%
Among community type	2	14.851	7.426	0.208	6%
Among sites	8	38.952	4.869	0.296	8%
Within sites	66	206.625	3.131	3.131	86%
Total	76	260.429		3.635	100%

d) Molecular variance of sites classified by disturbance level.

Source	df	SS	MS	Est. Var.	%
Among disturbance class	3	15.381	5.127	0.047	1%
Among Sites	7	26.687	3.812	0.115	3%
Within Sites	66	218.360	3.308	3.308	95%
Total	76	260.429		3.470	100%

Table 3.7: Identical and unique Australian AMV CP nucleotide sequences from naturalised *T. repens*. For comparison the CP insert used for GM *T. repens* was included (highlighted in grey). The GM insert does not share 100% sequence identity with any of the AMV CP sequences obtained from naturalised *T. repens*.

Groups of identical sequences														Unique sequences
Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10	Group 11	Group 12	Group 13	Group 14	
Carinya21_S1	Carinya30	Cumnock1_13	Carinya1	Nund14	Carinya25	Carinya27_S1	Bren1_6_S1	Coolah2_11	Castletop1_3	Pep1.2	Carinya19	Carinya10	Bren1_6_S2	Canob1_2
Coolah2_13	Carinya39	Cumnock2_6	Carinya8	Orange3	INR24_S3	Coolah2_22_S3	Coolah2_18	Coolah2_12	GI3#2_14	Pep1.8	Carinya22	Carinya13	Canob2_13	Canob2_13_S1
									Stoney1_10	Pep1.9	Carinya38	Carinya18	Carinya15	Carinya16_S1
										Pep1.11	INR24_S2	Coolah2_13	Carinya16_S2	Carinya24
												GI4_1	Carinya21_S2	Carinya26
												INR24	Carinya27_S2	Carinya26
													Carinya7	Carinya28
													Coolah1_19	Carinya29
													Coolah2_10	Carinya31
													Coolah2_22_S1	Carinya32
													Coolah2_22_S2	Carinya33
													Coolah2_23	Carinya34
													Coolah2_3	Carinya37
													Coolah2_5	Carinya5
													Coolah2_6	Carinya7_S1
													Coolah2_7	Cast10
													Coolah2_8	Cast14
													Coolah2_9	Cast22
													INR11	Coolah2_1
													LlanF1_9	Coolah2_19
													Terry4	Cumnock1_14
													Terry6	Hon
														INV5
														Orange12
														Pep1.1
														Pep1.10
														GM_insert

with PhiRT = 0.013 (P = 0.38), PhiPR = 0.034 (P = 0.22) and PhiPT = 0.047 (P = 0.08); Nm = 10.2.

3.3.1.1 Recombination

No recombination was detected using the default settings in SplitsTree4, SNAP workbench, GENECONV or Recco software. However a minimum of eight recombination events in the history of the sequences was detected by DnaSP software. Recombination was detected between nucleotide sites 3 and 153; 230 and 249; 303 and 315; 332 and 342; 342 and 376; 381 and 402; 402 and 411; and 411 and 435 (see Appendix 3.1 for alignment). A recombination rate (R) per sequence of 18.5 and 0.032 between adjacent sites was also estimated by the software. In addition recombination was detected by RAT software using the default settings (all sequences allowed to contribute), however no recombination was detected when the number of sequences allowed to contribute was restricted to a maximum of five. Restricting the number of sequences allowed to contribute to a recombination event was suggested on the RAT homepage (http://cbr.jic.ac.uk/dicks/software/RAT/index.html). As it is unlikely that many sequences contribute to recombination in any one sequence, and as the software uses homology to identify recombination, closely related sequences, as in the case of the AMV CP sequences analysed (sequence identity from 97.2 to 100%), may influence the results of the recombination test. Variants in this AMV population may, indeed, have undergone recombination; however the majority of recombination analysis programs used did not detect recombination. Therefore, for the purpose of neutrality tests, and for compiling phylogenetic trees for this study, I will assume no recombination.

3.3.1.2 Selection

Fu and Li's (1993) D* and F* and Tajima's D (1989) statistics were calculated, although it is possible that the assumption of no recombination was not met (see the section on recombination above). If no recombination is assumed Fu and Li's D* test statistic was -2.62 ($P < 0.05$), F* test statistic was -2.71 ($P < 0.05$)

and Tajima's D statistic was -1.64 (P>0.05). If there was recombination it is likely that the results gained may be more conservative than those in the absence of recombination (Ramos-Onsins and Rozas, 2002).

3.3.1.3 Spatial genetic structure and host association

Phylogenetic analysis of the 83 AMV sequences, depicted in rooted and unrooted nucleotide phylograms (Figures 3.2 and 3.3 respectively), and the haplotype network (Figure 3.4), indicate that AMV infecting naturalised *T. repens* in SE Australia exhibits no obvious geographically associated structure as there was little grouping of sequences by site or region in either phylogram, indicating a single undifferentiated population. However, any recombination events would impact the distribution of isolates on a phylogenetic tree as such trees only account for the variability observed between sequences due to nucleotide substitutions. Principal coordinates analysis (PCA) indicated that there was some population structure; however, this clustering was not explained by the geographic location of sites (Figure 3.5). A Mantel test was also undertaken to test for any correlation between geographic and genetic distance of AMV in SE Australia. No significant regression was observed ($R^2 = 0.0036$ $P = 0.17$), indicating no isolation by distance.

The collected AMV nucleotide sequences were compared to international AMV amino acid sequences from GenBank (Appendix 3.2). When sequences were compared in a rooted phylogram based on country of collection (Figure 3.6) there was clear grouping of all but one sequence from Canada but most sequences from other countries, including the Australian sequences, were distributed throughout the tree. When sequences were compared by host in a rooted phylogram (Figure 3.7) there was no clear grouping of sequences. It appears that AMV variants do not have a strict host association, as AMV isolated from *T. repens* can be found distributed throughout the tree.

Figure 3.2: Rooted NJ phylogram depicting the relationships between the AMV isolates collected from infected naturalised Australian *T. repens* and the coat protein insert (GM_insert) used for GM AMV-resistant *T. repens,* based on the alignment of the nucleotide sequence of the CP gene. The sequence used for transgenic clover (GM_insert) is contained within a red box. The bar indicates the relative evolution distance. Bootstrap values >75 are displayed on branches.

Figure 3.3: Unroooted NJ phylogram depicting the relationships between the AMV isolates collected from infected naturalised Australian *Trifolium repens* and the coat protein insert (GM_insert) used for transgenic clover, based on the alignment of the nucleotide sequence of the CP gene. The sequence used for transgenic clover (GM_insert) is contained within a red box. The bar indicates the relative evolution distance and bootstrap values are displayed on tree branches.

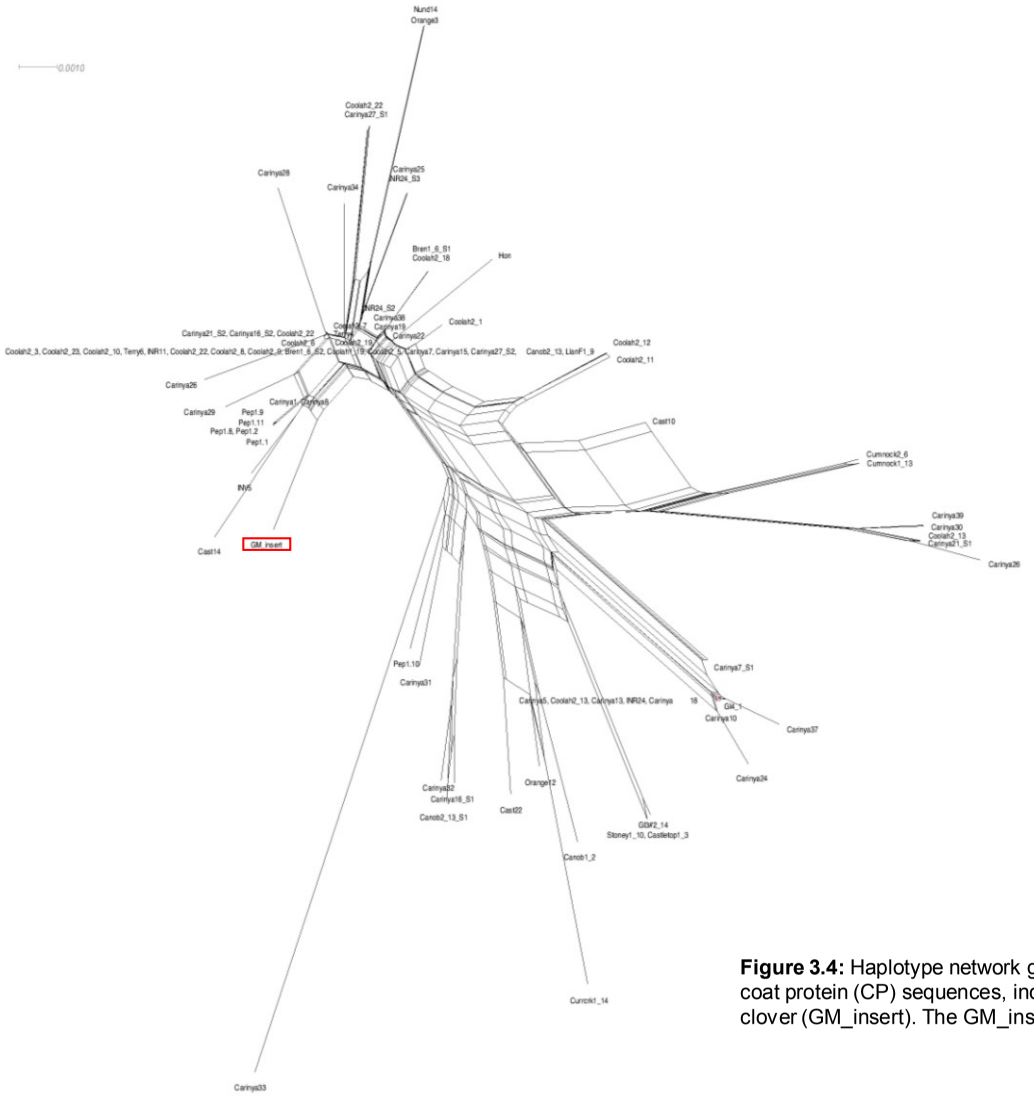


Figure 3.4: Haplotype network generated in SplitsTree of Australian AMV coat protein (CP) sequences, including the CP used for transgenic white clover (GM_insert). The GM_insert is contained within a red box.

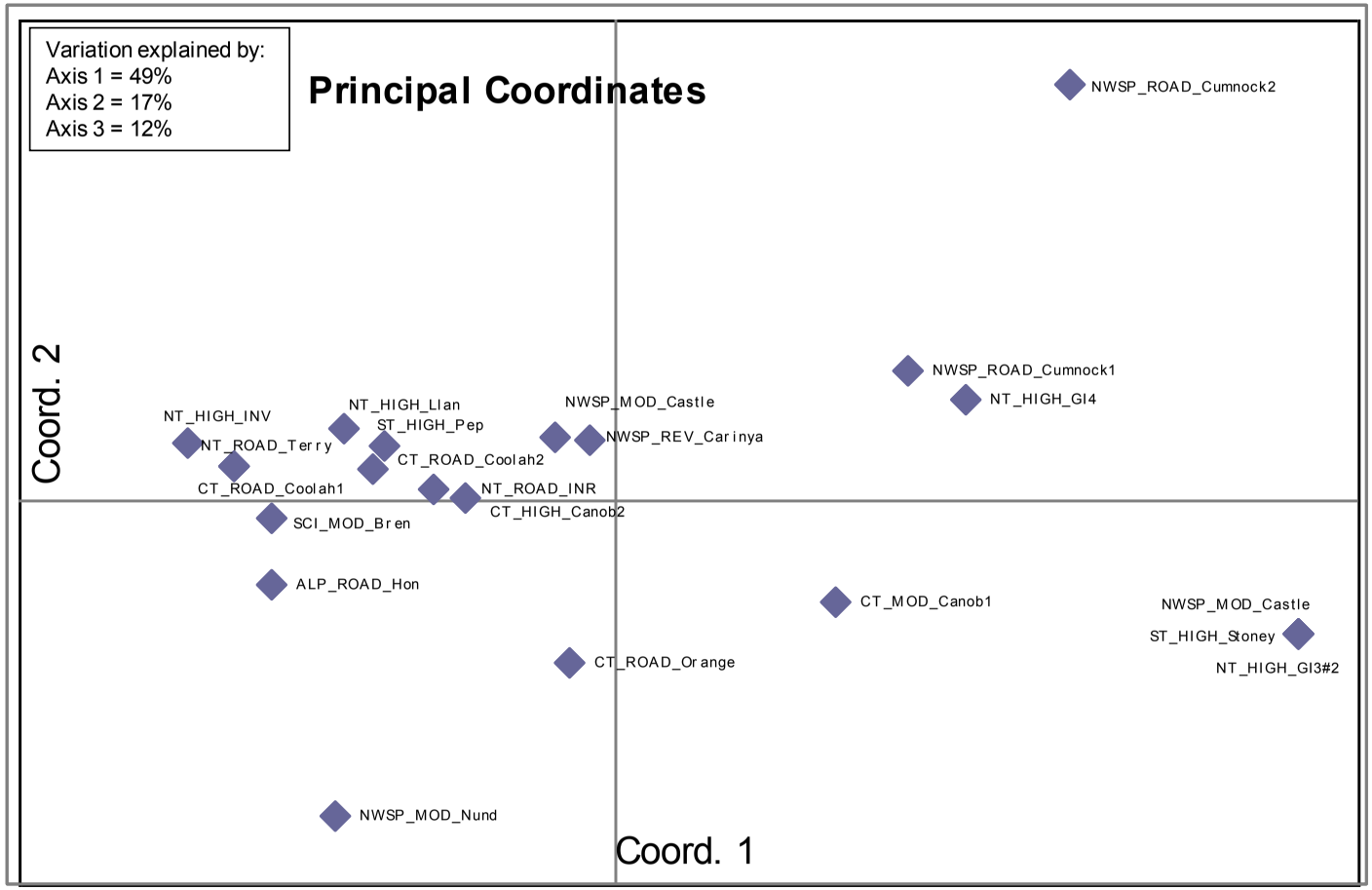


Figure 3.5: Principal coordinates analysis (PCA) of Australian AMV CP sequences grouped by site. Site names include geographic region code_community type code_site name. Geographic regions include Central Tablelands (CT), North West Slopes and Plains (NWSP), Northern Tablelands (NT), Southern Tablelands (ST) and South Coast and Illawarra (SCI) NSW. Community types include roadside (ROAD), revegetation site (REVEG), communities of moderate conservation value (MOD) and communities of very high conservation value (HIGH).

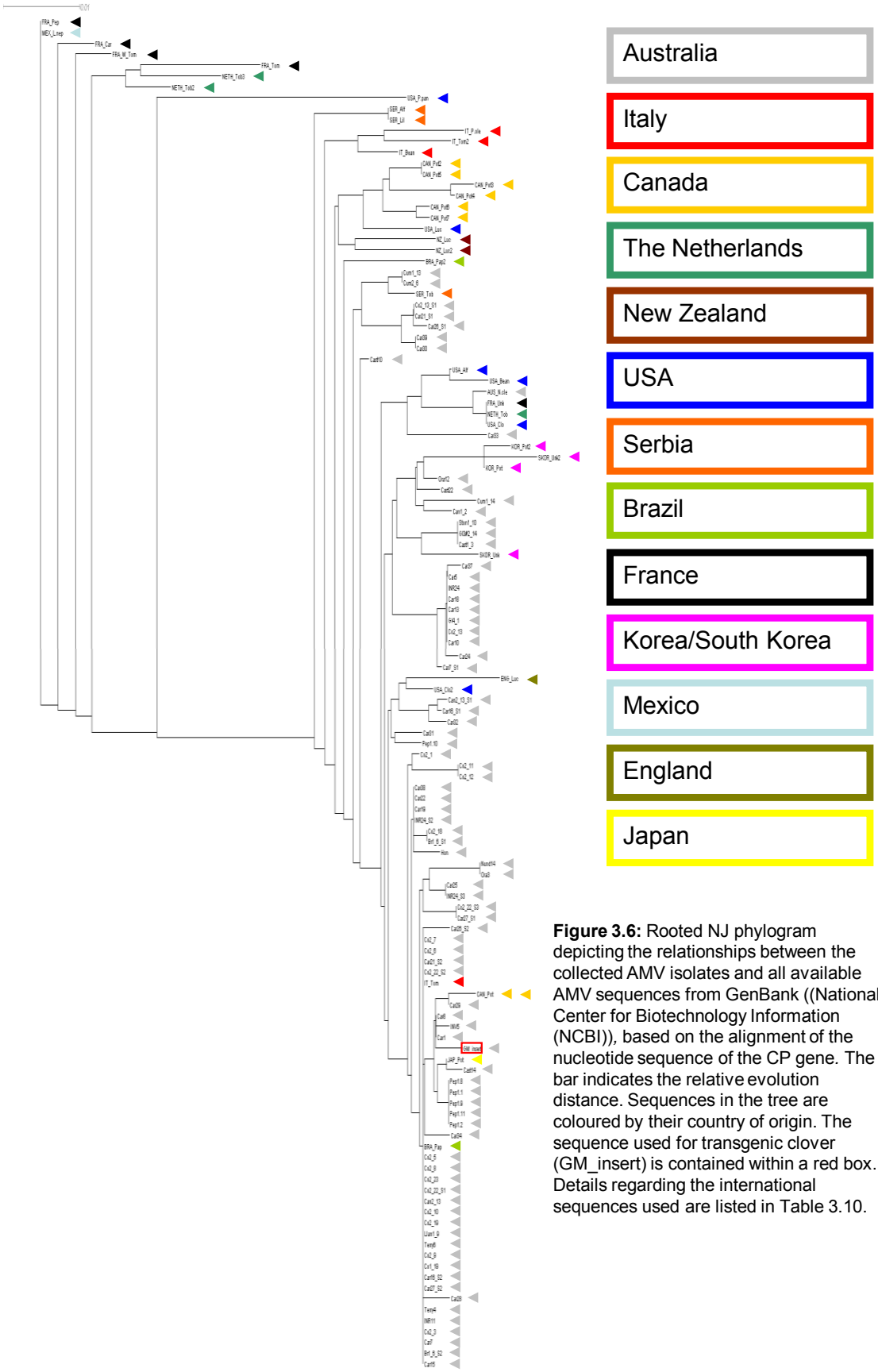


Figure 3.6: Rooted NJ phylogram depicting the relationships between the collected AMV isolates and all available AMV sequences from GenBank ((National Center for Biotechnology Information (NCBI)), based on the alignment of the nucleotide sequence of the CP gene. The bar indicates the relative evolution distance. Sequences in the tree are coloured by their country of origin. The sequence used for transgenic clover (GM_insert) is contained within a red box. Details regarding the international sequences used are listed in Table 3.10.

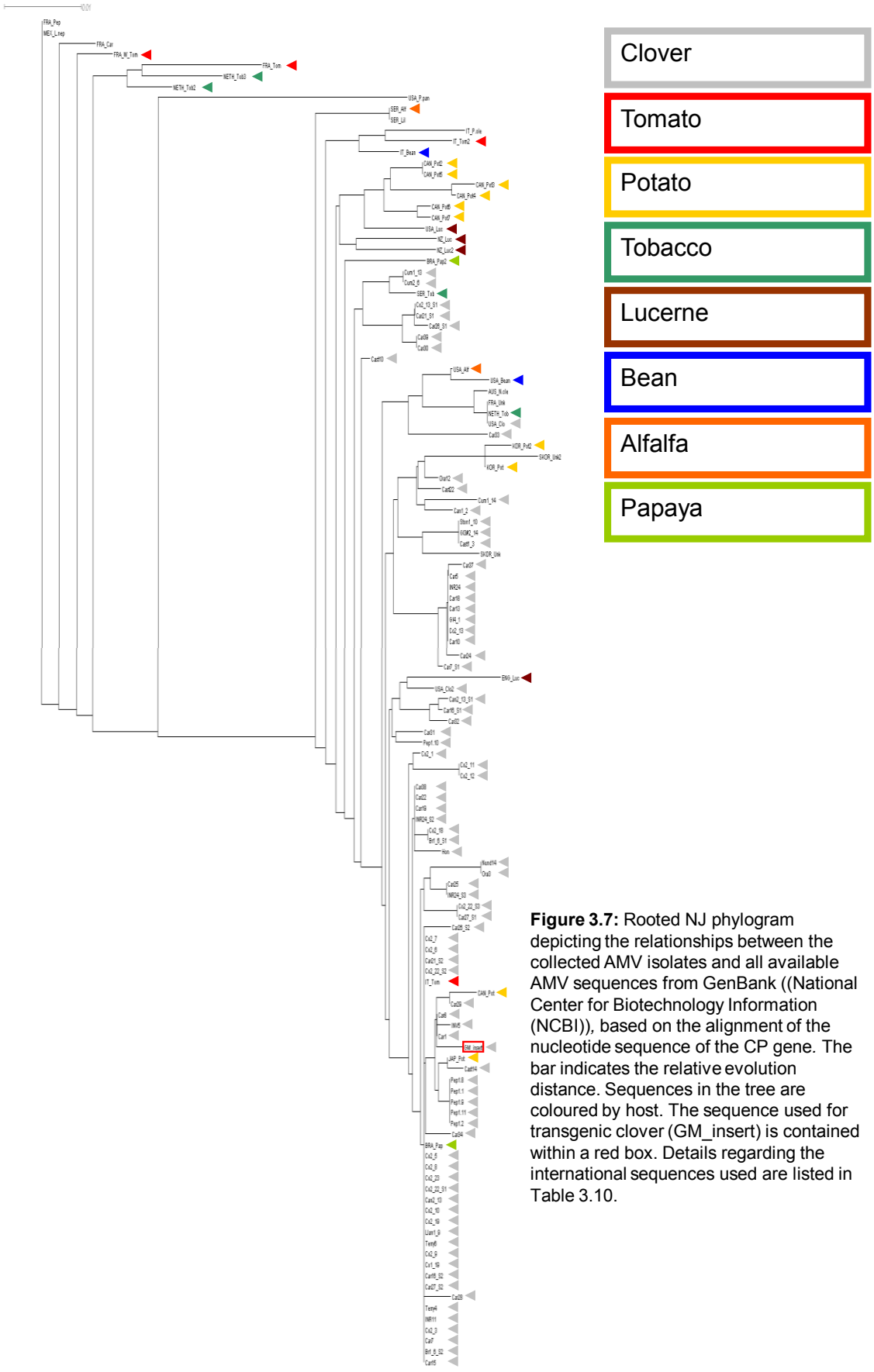


Figure 3.7: Rooted NJ phylogram depicting the relationships between the collected AMV isolates and all available AMV sequences from GenBank ((National Center for Biotechnology Information (NCBI)), based on the alignment of the nucleotide sequence of the CP gene. The bar indicates the relative evolution distance. Sequences in the tree are coloured by host. The sequence used for transgenic clover (GM_insert) is contained within a red box. Details regarding the international sequences used are listed in Table 3.10.

3.3.2 Comparison of Australian Alfalfa mosaic virus to the coat protein insert used for genetically modified Trifolium repens

None of the Australian AMV sequences had a nucleotide sequence identical to the GM_insert (Table 3.8 and alignment in Appendix 3.1). The sequence identity between the GM_insert and AMV sequences obtained in this study ranged from 97.7% (*Cumnock1_14*) to 99.6% (*Carinya1*). The closest relatives to the GM_insert were sequences from sites *Pep*, *Carinya*, *INV* and *Cast* (Figures 3.2, 3.3 and 3.4). Of the AMV isolates 71% (59 of 83) had an amino acid sequence (193 amino acids) identical to the CP sequence used for GM *T. repens* (Table 3.8). The remainder had at least one amino acid difference or at least one unknown amino acid (due to an ambiguous base in the original nucleotide sequence) (Table 3.8). The greatest difference in amino acid composition was displayed by sequences *Carinya33* and *Carinya26* which had four amino acid differences compared to the GM_insert. The nature of the amino acid differences between the GM_insert and the collected sequences varied. There were six types of substitutions: Threonine (T) to Isoleucine (I) (not conservative); Valine (V) to Alanine (A) (conservative); A to V (conservative); Glutamine (Q) to Histidine (H) (not conservative); A to T (not conservative); and Glycine (G) to Glutamic acid (E) (not conservative).

3.3.2.1 Predicted secondary protein structure

The secondary protein structure of the collected sequences and the GM_insert was predicted based on the primary amino acid sequence (see Appendix 3.3 for the sequence alignment). Table 3.9 displays a schematic representation comparing the predicted secondary structure of the GM_insert to the collected sequences that had at least one known amino acid difference to the GM_insert (see Table 3.8 for the specific amino acid differences). All of the amino acid substitutions observed resulted in a change in the predicted secondary protein structure. All changes occurred between amino acid position 44 and 163, with the majority between amino acid 59 and 112.

Table 3.8: Comparison of the amino acid sequence of the CP insert used for GM *Trifolium repens* (GM_insert) and Australian AMV. Aligned nucleotide sequences were translated to amino acid sequences with the appropriate reading frame for the AMV CP. Identical amino acid sequences, sequences with possible differences (X) and sequences different to the GM_insert are listed.

Identical to GM_insert	With ≥1 unknown amino acid (X)			With ≥1 different amino acids		
Sequence	Sequence	Substitution[a]	Amino acid position	Sequence	Substitution[a]	Amino acid position
Bren1_6_S1	Pep1.1	G→X	82	Carinya26	T→I	77
Bren1_6_S2	Carinya5	K→X	138	Canob1_2	V→A	111
Canob2_13				Carinya32	V→A	111
Carinya1				Cast22	V→A	111
Carinya10				Castletop1_3	V→A	111
Carinya13				GI3#2_14	V→A	111
Carinya15				Stoney1_10	V→A	111
Carinya16_S2				Carinya25	A→V	151
Carinya18				INR24_S3	A→V	151
Carinya19				Carinya21_S1	Q→H, V→A & A→T	109, 111 & 126
Carinya21_S2				Carinya30	Q→H, V→A & A→T	109, 111 & 126
Carinya22				Carinya39	Q→H, V→A & A→T	109, 111 & 126
Carinya24				Coolah2_13	Q→H, V→A & A→T	109, 111 & 126
Carinya27_S1				Cumnock_14	V→A & A→T	111 & 126
Carinya27_S2				Cumnock1_13	V→A & V→A	17 & 111
Carinya28				Cumnock2_6	V→A & V→A	17 & 111
Carinya29				Carinya33	V→A, V→A, A→V & A→T	17, 48, 59 & 114
Carinya31				Cast10	V→X, S→X, V→A, D→X & F→X	17, 75, 111, 150 & 173
Carinya34				Canob2_13_S1	G→E & V→A	45 & 111
Carinya37				Carinya16_S1	G→E & V→A	45 & 111
Carinya38				Carinya26	T→I, Q→H, V→A & A→T	77, 109, 111 & 126
Carinya7				Orange12	H→X & V→A	80 & 111
Carinya7_S1						
Carinya8						
Cast14						
Coolah1_19						
Coolah2_1						
Coolah2_10						
Coolah2_11						
Coolah2_12						
Coolah2_13						
Coolah2_18						
Coolah2_19						
Coolah2_22_S1						
Coolah2_22_S2						
Coolah2_22_S3						
Coolah2_23						
Coolah2_3						
Coolah2_5						
Coolah2_6						
Coolah2_7						
Coolah2_8						
Coolah2_9						
GI4_1						
Hon						
INR11						
INR24						
INR4_S2						
INV5						
LlanF1_9						
Nund14						
Orange3						
Pep1.10						
Pep1.11						
Pep1.2						
Pep1.8						
Pep1.9						
Terry4						
Terry6						

[a]Most common amino acid at that position in alignment→replacement amino acid (one letter amino acid code).

Table 3.9: A schematic representation of the predicted protein secondary structure of the transgenic *Trifolium repens* coat protein (CP) insert (GM_insert) and the predicted three dimensional structure of Australian AMV CP sequences from naturalised *T. repens*. The secondary structure, coil (C), extended-beta (E) and helix (H), of the proteins were predicted based on the primary amino acid sequence (Arnold *et al., 2006)*. Only sequences that were different to the GM_insert are displayed. Specific amino acid differences in the sequences are listed in Table 3.8.

	Amino acid position																										
Sequence	1~11	12~14	15	16	17	18~29	30~33	34~37	38~40	41~43	44	45	46~58	59	60	61	62	63	64~73	74	75	76	77~82	83~87	88~96	97~99	100~103
GM_insert	C	E	E	E	E	C	H	C	E	C	C	C	H	C	C	C	C	C	H	H	E	E	C	E	C	E	C
Carinya26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	H	-	-	-	-	-
Canob1_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cast22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Castletop1_3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GI3#2_14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stoney1_10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cast10*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OrangE12*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
INR24_S3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
Carinya16_S1	-	-	-	-	-	-	-	-	-	-	H	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Canob2_13_S1	-	-	-	-	-	-	-	-	-	-	H	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
Carinya39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
Coolah2_13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
Carinya21_S1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
Cumnock1_14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
Cumnock1_13	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
Cumnock2_6	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
Carinya33	-	-	-	C	C	-	-	-	-	-	-	-	-	H	-	H	H	H	-	E	-	-	-	-	-	-	-
Carinya26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	H	-	-	-	-	-

Table 3.9: continued

	Amino acid position																								
Sequence	104~106	107	108	109	110	111	112	113~119	120~125	126~138	139~148	149	150~160	161	162	163	164	165	166~169	170	171	172~178	179~186	187~193
GM_insert	H	C	C	C	H	H	H	C	E	C	E	C	C	C	C	H	H	H	E	H	H	C	H	C
Carinya26	-	H	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Canob1_2	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya32	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cast22	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Castletop1_3	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GI3#2_14	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stoney1_10	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cast10*	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OrangE12*	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya25	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-
INR24_S3	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-
Carinya16_S1	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Canob2_13_S1	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya30	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya39	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coolah2_13	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya21_S1	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cumnock1_14	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cumnock1_13	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cumnock2_6	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carinya33	-	-	-	-	-	-	-	-	-	-	-	-	-	E	E	E	-	-	-	-	-	-	-	-
Carinya26	-	-	-	-	C	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*The SWISS-MODEL Workspace didn't recognise the amino acid code X (unknown amino acid) in sequence Cast10 and Orange12, therefore for analysis X was replaced with the most common amino acid at that position in the other sequences.

3.4 Discussion

I have determined and compared the RNA 3 CP sequence of 83 AMV isolates (nucleotide sequence identity 97.2 to 100%) from 19 SE Australian naturalised *T. repens* populations in order to assess the population and spatial structure of AMV in SE Australia. Although there were 49 polymorphic sites, no obvious associated genetic or geographic structure could be determined. The source country, or host, of SE Australian AMV could not be ascertained as Australian sequences did show close genetic association with sequences from a particular country or host. No Australian AMV CP nucleotide sequence was identical to the GM_insert used for transgenic *T. repens*, with sequence identity ranging from 97.7 to 99.6%, and although the majority (71%) of the isolates shared the same amino acid sequence as the GM_insert, 29% contained up to four amino acid differences.

3.4.1 Alfalfa mosaic virus from naturalised Trifolium repens in south-eastern Australia

3.4.1.1 Genetic diversity

It has been argued that RNA viruses exhibit high mutation rates, thought to be the highest for any organism (Moya *et al.*, 2000). Point mutations occurring during the replication of RNA viral genomes are believed to be the predominant source of diversity (Ramirez, 1995). Mutations are estimated to appear in RNA genomes at a rate of about 10^{-3} per nucleotide position per replication cycle (Gibbs *et al.*, 1995), although direct estimates of plant virus mutation rates are rare (Sanjuán *et al.*, 2009). Mutation rates are thought to be high because RNA viruses tend to have large populations, high replication rates and short generation times potentially resulting in high genetic variability (Moya *et al.*, 2000). In spite of this argument, RNA viruses tend to have a very efficient use of genetic material in their genome, with a small number of non-coding regions and coding sequences that are closely packed together. In the case of viruses such as AMV, coding regions for genes may overlap and genes may be completely contained within one another. Single gene products may

also have multiple functions, for example the AMV CP, responsible for protection of the genome, also has functions in insect transmission, vector specificity, cell-to-cell movement, the expression of symptoms and potentially the control of replication (Hull, 2002). With multiple functions being performed by parts of a small genome and a lack of redundancy, any nonsynonymous polymorphism (resulting in an amino acid replacement) could reduce virus fitness or survival. As current estimates of mutation rate available for RNA plant viruses are low, for example 3×10^{-5} per site and round of replication for *Tobacco etch virus* (TEV), it is possible that selection pressures unique to plant viruses such as strong host driven bottlenecks have driven RNA plant viruses towards lower mutation rates than other types of RNA viruses (Sanjuán *et al.*, 2009).

A CP gene nucleotide diversity (Pi) of 0.009 (Table 3.1), sequenced from 83 AMV isolates collected from 19 sites across NSW, was lower than expected for an RNA virus (Moya and García-Arenal (1995) consider a Pi of 0.01-0.03 as low). RNA viruses such as influenza A and hepatitis C have been well studied and, although these viruses are highly variable, the nucleotide diversity of SE Australian AMV is consistent with other studies of RNA viruses with plant hosts which generally display low variability (García-Arenal *et al.*, 2001). An exception is *Rice yellow mottle virus* which evolves as rapidly as a large proportion of animal RNA viruses (Fargette *et al.*, 2008).

As low Pi is likely to be an indicator of high genetic stability (Sánchez-Campos *et al.*, 2002), then high genetic stability, compared to animal RNA viruses, may be the norm for plant RNA viruses (Sanjuán *et al.*, 2009). No indels (insertions or deletions) were observed when comparing the Australian AMV sequences and the AMV CP appears to be conserved with 59 (71%) of the 83 amino acid sequences found to be identical.

3.4.1.2 Population structure

If AMV was dispersed in the landscape by natural means only (aphids), it would be expected to observe a clear genetic population structure by site and region, leading to genetic isolation by distance. All biological species must exhibit some geographically associated structure, i.e. not completely random in pattern and spatial distribution (Hartl, 2000). However, within the collected Australian AMV CP nucleotide sequences there was little obvious population structure or isolation by distance. There appeared to be low association between genetic diversity and collection site (very few sequences from the same grouped together on phylogenetic trees), plant community type or conservation value of the community (Tables 3.1, 3.2 and 3.4; Figures 3.2, 3.3, 3.4 and 3.5). Geographic location appears to have some influence on Pi. Sequences from the South Coast and Illawarra region (site *Bren*), had a lower Pi than other regional groups (Table 3.3), possibly due to the geographic separation from other AMV infected sites. Although sites with high disturbance are often roadsides and may contain other sources of AMV infection (other naturalised host species), high disturbance was not necessarily correlated with high Pi, in this survey the group of sequences from sites with high disturbance had a lower Pi than those with low or medium disturbance (Table 3.5). Although these results indicate that there is some genetic structure, based on geographic location and site disturbance level, on the whole there was very little obvious genetic structure of the SE Australian AMV population. This result may be an indicator of genetic stability within the population (Lynch and Crease, 1990), and/or reflect anthropogenic dispersal of AMV in the landscape.

Given that AMV is not seed transmitted in white clover (Jones and Pathipanawat, 1989), and there are many potential AMV hosts found in agricultural landscapes (Appendix 2.3), it is likely that agricultural species are a source of AMV, which is then transported short distances by aphids to *T. repens*

in nearby native plant communities. Previous work demonstrated that sites close (<1 km) to cropping or *T. repens* pasture are significantly more likely to be infested with AMV than sites further away from cropping or *T. repens* pasture (>1 km) (Chapter 2), indicating that human activity is likely to impact AMV population structure in naturalised *T. repens* in habitats close to agriculture. Sequence analysis in this chapter, spatial analysis (PCA), isolation by distance analysis (Mantel test) and AMOVA, indicate a general lack of population structure of AMV in SE Australia. These results appear to confirm that human activity is influencing AMV populations in the habitat types sampled. It is likely that the importation and distribution of pasture species in Australia has facilitated the introduction/s of AMV into naturalised *T. repens* populations present in native plant communities. It follows that the dispersal of AMV through repeated circulation of infected pasture seed or plant material in the agricultural landscape has resulted in a lack of associated population structure of AMV in naturalised *T. repens*.

3.4.1.3 Selection

Although it can be difficult to distinguish between the influence of genetic drift and selection on an RNA virus population, genetic drift, specifically founder effects, result in a lower diversity within a site and a larger diversity between sites (García-Arenal *et al.*, 2001). The opposite was found for the SE Australian AMV population, more diversity was detected within sites than between sites (Table 3.6), indicating that selection may more realistically explain the population genetic structure.

For populations that conform to the neutral mutation model it is expected that statistical tests of the neutrality of mutations equate to zero or close to zero (Innan and Wolfgang, 2000). Significantly negative values, as in the case of SE Australian AMV, can indicate an abundance of low-frequency variants, resulting from population expansion, positive selection or weak negative selection (Barreiro and Quintana-Murci, 2010). A ω ratio of less than one, as in

the case of the SE Australian AMV population (ω=0.075), signifies the population is undergoing purifying (negative) selection (Yang *et al.*, 2000), indicating that less fit variants are decreasing in frequency within the population (García-Arenal *et al.*, 2001). For most RNA plant viruses studied to date selection has proven to be negative (García-Arenal *et al.*, 2001). Given the difficultly of mechanical inoculation of AMV onto *T. repens* under experimental conditions (see Chapter 4), AMV is likely to be strongly reliant on aphid vectors for distribution in natural and agricultural systems in SE Australia. While the factors influencing selection can be difficult to determine, there is strong evidence for vector-associated negative (purifying) selection of plant RNA viruses in many cases (García-Arenal *et al.*, 2001), which may also be the case for the SE Australian AMV population.

3.4.1.4 Population origin and size

When the CP sequences of the Australian AMV isolates were compared to the international CP sequences available, by collection location or host (Figures 3.6 and 3.7), Australian sequences grouped with sequences from many countries and host types. This is not unexpected as plant virus sequences from distant geographic locations are often found to be closely related, an effect likely to be a result of the world trade of infected plant material (Moya *et al.*, 1993). From my results I cannot ascertain the geographic location from which Australian AMV was sourced. It is possible that AMV was introduced numerous times into Australia from different locations, as has been demonstrated for many other plant pathogens (Moya *et al.*, 1993). A lack of grouping of any sequences by host on the phylogenetic trees indicates that particular AMV isolates are not necessarily linked to any host type and that host shifting events in AMV appear to be frequent.

3.4.2 *Potential efficacy and durability of coat protein-mediated resistance to south-east Australian Alfalfa mosaic virus*

A number of hypotheses have been suggested to explain the CP-mediated resistance mechanism. The most commonly accepted method for AMV is that the CP insert could inhibit virons from undertaking co-translational disassembly which occurs early in plant infection (Timmerman-Vaughan *et al.*, 2001, Lin *et al.*, 2007). As soon as the invading virus liberates its 5' terminal CP subunit, ready for translation of the viral genome, the CP produced from the transgene instantly recoats any disassembling virus and stops infection (Lin *et al.*, 2007). Resistance to AMV was only detected in transgenic peas with an AMV CP insert when CP product was present (Timmerman-Vaughan *et al.*, 2001). Therefore resistance in *T. repens* is likely to rely on the production of CP product from the introduced CP gene, rather than RNA-mediated resistance (an alternative hypothesis for the resistance mechanism). As a consequence, the amino acid sequence expressed by the transgenic plant is crucial for resistance. If the CP insert used for GM *T. repens* is not highly similar to the CP of AMV isolates currently present in SE Australia then AMV variants may already exist that can overcome resistance. In addition, the long term durability of resistance is likely to be strongly negatively correlated with the evolutionary potential of the virus (recombination, gene flow and population size) (García-Arenal and McDonald, 2003). Although the nucleotide diversity of AMV in SE Australia is relatively low, the evolutionary potential of AMV in SE Australia appears moderate to high given the high gene flow, potential for recombination (data for recombination ambiguous), large potential population size (many possible host species in Australia (see Appendix 2.3)) and the high number (27%) of AMV isolates with a different amino acid sequence to the GM_insert (Table 3.8).

3.4.2.1 Efficacy of resistance

South-eastern Australian AMV sequences were compared to the CP sequence used for GM *T. repens* (GM_insert) and although none had a nucleotide sequence identical to the GM_insert, 71% had an identical amino acid sequence, most likely rendering those isolates avirulent on GM clover. It is unknown how different the CP sequence needs to be before CP-mediated resistance breaks down. It has been documented in numerous cases that virus resistance genes have been overcome by viruses with four or less amino acids changes (Harrison, 2002). Taschner *et al*. (1994) demonstrated that a CP transgene sourced from an AMV mutant, with only one amino acid difference, was unable to provide resistance for transgenic tobacco against wild-type AMV. Two of the AMV CP sequences I collected had four amino acid differences compared to the GM_insert (Table 3.8); it is plausible that these variants could overcome resistance conferred by the GM_insert. In addition the lack of population structure displayed by AMV in this system, with the majority of genetic diversity within sites (≥83%), it is likely that if released in SE Australia the transgenic plants will be rapidly exposed to the full suite of AMV variation observed.

It is unknown how important the nature of the amino acid substitution is in AMV resistance breakdown. Bendahmine *et al.* (1999) demonstrated that transgenic tobacco plants expressing a *Tobacco mosaic virus* (TMV) CP mutant, resulted in a protein product that was unable to aggregate and displayed low resistance to TMV. Also, transgenic tobacco containing an AMV CP with a frameshift mutation was susceptible to infection (Van Dun *et al.*, 1988). For various fungal effector proteins, single amino acid changes can result in a change from avirulence to virulence. However, in most cases, it is the position of the amino acid substitution in relation to the protein structure that determines whether the substitution results in a virulence reaction on the host (Dangl and Jones, 2001, Joosten *et al.*, 1994, Schürch *et al.*, 2004).

Nonsynonymous polymorphisms result in an amino acid replacement and, depending on the type of amino acid substituted (basic, acidic, polar or non-polar), the secondary structure and functional ability of the protein could be dramatically changed. Twenty-two of the collected AMV sequences had at least one amino acid difference compared to the GM_insert. When the secondary structure of the proteins was predicted, based on primary amino acid sequences, there were varying degrees of structural changes to all of the collected sequences that had at least one amino acid difference. It is difficult to predict the impact this may have on resistance conferred by the GM_insert. Perhaps the use of a model such as that described by Fabre *et el.* (2009) may provide more insight into the durability of CP-mediated resistance in this system, however prior to commercial release it is vital that AMV-resistant transgenic white clover is exposed to AMV isolates representing the CP diversity present in SE Australia to confirm the durability of resistance and the risk to non-target habitats.

3.4.2.2 Durability of resistance

Bosch *et al.* (1986) demonstrated that resistance that is facilitated by the vector, or resistance resulting in a reduction in the inoculation of the plant (reduced plant exposure to virus) does not place the virus under selection pressure to evolve an increased multiplication rate. In contrast, within-plant resistance, as in the case of GM *T. repens*, facilitating a reduction of virus titre, or a reduction in symptoms, places the virus under a selection pressure to evolve an increased multiplication rate and could result in an increase in genetic diversity of the virus population. However, other factors may restrict this increase in diversity. It is thought that resistance genes against viruses may in fact prove more durable than those for other plant pathogens (García-Arenal and McDonald, 2003). The effective population size (virus particles that actually come in contact with each other) may be relatively low compared to plant pathogenic fungi or bacteria (García-Arenal and McDonald, 2003), although the effective

population size could be considerable for a virus given the wide host range of AMV. In addition, the conservative nature of viral genomes is likely to restrict the nature of mutations while still maintaining fitness.

Currently a group of AMV variants in SE Australia may already contain enough CP diversity to breakdown resistance. However, the long term durability of resistance is difficult to ascertain. Janzac *et el.* (2009) used a model to predict the durability of resistance genes based on the evolutionary constraints impacting avirulence factors. They found a strong relationship between ω ratio and durability of resistance, the lower the ω ratio value the higher the durability of resistance. In addition a marginally significant relationship ($P = 0.052$) was found between nucleotide diversity and durability, the higher the diversity the higher the durability of resistance (Janzac *et al.*, 2009). The ω ratio (0.075) and nucleotide diversity (0.009) of SE Australian AMV compared to those viruses in the study were both low, therefore it is difficult to place AMV into a discrete durability class and, as a consequence, I am unable to theoretically predict the resistance durability of CP-mediated resistance to AMV in *T. repens*. As theoretically the long term durability of resistance cannot be ascertained, the exposure of the transgenic line to AMV isolates representing the variation observed in SE Australia is required.

3.4.2.3 The potential impact of co-infecting viruses

Naturalised *T. repens* in SE Australia is often found infected with AMV and at least two other virus species, *Clover yellow vein virus* (ClYVV) and *White clover mosaic virus* (WClMV) (Chapter 2). It is not yet understood how Australian AMV interacts in *T. repens* with other viruses or with the GM_insert. Viruses infecting the same host have been demonstrated to work synergistically to interfere with plant resistance genes (García-Cano *et al.*, 2006). Complementation has been illustrated for AMV in other plant species. It has been demonstrated that, for cell-to-cell movement, AMV is able to utilise movement proteins from the same family *Bromoviridae* (Sanchez-Navarro *et al.*,

2006). CPs from *Alfamoviruses* and *Ilarviruses* can be interchanged resulting in successful binding and genome activation (Tenllado and Bol, 2000). In addition, RNA 3 of the AMV genome can be complemented in plants transformed with AMV RNA 3 (Van Der Kuyl *et al.*, 1991, Van der Vossen, 1996). Although this thesis cannot address this aspect, the nature of AMV interactions with co-infecting viruses and the GM_insert in transgenic *T.* repens is likely to be important for long-term resistance durability and the environmental risk assessment.

3.4.3 Implications for the risk assessment

If GM *T. repens* is found to be resistant to AMV and the transgene is found to provide a competitive advantage to naturalised *T. repens*, then native plant communities in SE Australia are likely to be at risk from further invasion by *T. repens.* In contrast, if AMV can overcome resistance or other factors reduce resistance such as the presence of other viruses, then the environmental consequences of a commercial release of the transgenic are likely to pose a low risk to native habitats.

3.4.4 Conclusion

When the GM_insert was compared to the sequences collected, 71% of the AMV isolates studied possessed an identical amino acid sequence to the GM_insert, implying that the bulk of AMV isolates in SE Australia will be unable to infect GM *T. repens.* However, the CP of the remaining isolates differed to the GM_insert by up to four amino acids. The extent or nature of amino acid differences required to overcome resistance in GM *T. repens* demands further research. In addition, the AMV isolates collected appear to have no apparent geographically associated population structure, no clear origin or pattern of distribution in the landscape, no obvious country or host of origin and no observable association with particular hosts. Consequently, it is likely that AMV dispersal in SE Australia is largely human mediated. It is probable that the circulation of AMV infected seed and plant material in the

agricultural landscape has facilitated the dispersal of AMV in the area surveyed and will continue to act as a source of AMV. My results indicate that if transgenic *T. repens* is grown in the area surveyed is likely to be exposed to the full suite of AMV variants observed. It is crucial then that prior to the commercial release of transgenic AMV-resistant *T. repens,* research is conducted to test the resistance of GM *T. repens* to isolates representing the genetic diversity present in SE Australia.

4 Impact of *Alfalfa mosaic virus* on the growth and morphology of naturalised and cultivated *Trifolium repens*

4.1 Introduction

Gene flow from transgenic to non-transgenic plant species has been investigated intensively during the last decade, but the ecological impacts of transgene flow have been less extensively studied (Warwick *et al.*, 2009). There are two key ecological concerns regarding the environmental release of genetically modified (GM) *Alfalfa mosaic virus* (AMV)-resistant white clover (*Trifolium repens*). These are: i) possible escape and naturalisation of GM *T. repens;* and ii) transgene introgression into already naturalised *T. repens.* Either of these events may result in an increase in the abundance or distribution of clover in native plant communities (non-target habitats).

As an assessment of the weediness risk of a novel plant genotype can be difficult, Raybould and Cooper (2005) reason that risk assessment should begin with simple experiments replicating "worst case" scenarios, and become more complex as required if simpler studies do not indicate negligible risk (acceptable risk) with adequate certainty. Many publications recommend the use of "tiered risk assessment" for the assessment of transgenic plants (Wilkinson, 2003). Briefly, the process begins with the first tier which tests the "worst case scenario" under controlled conditions (lab or glasshouse). If results indicate that harm/exposure is negligible, then it can be concluded that risks are negligible. However, if there is concern regarding risk following tier one tests, then tier two studies are conducted. Tier two studies assess risk under more realistic conditions (field trials). Again, if harm/exposure is not demonstrated to be negligible, then tier three studies are undertaken (large scale field trials) (Wilkinson and Tepfer, 2009).

Studies conducted by Godfree *et al.* (2004b) and (2006) prior to the commencement of this risk assessment, demonstrated that AMV is a pathogen of naturalised *T. repens* and *T. repens* plants are a significant component of at least two high conservation-value habitat types in SE Australia. Given this information regarding the nature of the AMV-*T. repens* pathosystem, in this thesis the procedure used to assess the potential risk posed by transgenic AMV-resistant *T. repens* to non-target habitats in south-eastern (SE) Australia involves four stages: i) potential habitat identification; ii) field survey; iii) pathogen detection and development of host-pathogen (H-P) arrays; followed by iv) tiered risk assessment (see Figure 2.1). Stages one, two and part of three (pathogen detection) are considered in Chapters 2 and 3 of this thesis. This chapter addresses the remainder of stage three (development of H-P arrays) and part of stage four (H-P challenges and fitness effects).

Transgenic pasture species have been identified as posing an enhanced risk of escape compared to more domesticated crop species (Warwick *et al.*, 1999). This has been demonstrated in practice by the escape and naturalisation of GM creeping bentgrass (*Agrostis stolonifera* L.) in native plant communities within the USA (Reichman *et al.*, 2006). Warwick *et al.* (2009) proposes a list of characteristics that are likely to be associated with weediness risk, of which *T. repens* possesses many (Baker and Williams, 1987).

Trifolium repens has a recorded history of invasive success (Wu *et al.*, 2009) and has been shown to reduce species richness in native habitats (Warren, 2000). White clover can dominate unmanaged habitats and is currently regarded as a significant weed in a broad range of natural and modified environments in SE Australia, including high quality native plant communities (Chapter 2). AMV infection of *T. repens* is common in agricultural pastures in Australia (Norton and Johnstone, 1998, McKirdy and Jones, 1997, McKirdy and Jones, 1995, Coutts and Jones, 2002, McLean, 1983), and has also been detected in

naturalised *T. repens* in various habitats in SE Australia, although not as commonly as in agricultural pastures (Chapter 2).

Considering the distribution of *T. repens* and AMV in non-target habitats (Chapter 2), it is possible that this virus is currently playing a role in limiting the growth and expansion of *T. repens* populations in some native habitats in SE Australia. The prevalence of AMV, and the associated impact of AMV on naturalised host populations, determines the maximum potential for ecological release [the enemy-release hypothesis (Keane and Crawley, 2002)] to occur in non-target environments following the release of AMV-resistant clover lines.

The enemy release hypothesis argues that the impact of an invasive species is increased due to reduced attack by natural enemies (Keane and Crawley, 2002). This has been demonstrated by Mitchell and Power (2003), in that invasive plants in natural systems that are released from pathogens, including viruses, are more widely considered harmful. There is strong evidence confirming the negative impact of enemies on the fitness of native and naturalised hosts (Bock, 2008, Simelane and Phenye, 2005, Funayama *et al.*, 2001, Yahara and Oyama, 1993). In addition, the success of numerous biological control agents (Myers *et al.*, 2009, Barton *et al.*, 2007, McConnachie *et al.*, 2004), demonstrate the likely importance of enemies in host population dynamics. Numerous other studies have considered risk assessments of transgenics with virus resistance (Tepfer, 2002, Robinson, 1996), however few have considered virus-resistant transgenic plants that are known to be weedy (see Laughlin *et al.* (2009) and Raybould and Cooper (2005) for examples).

Trifolium repens and associated viruses comprise a good model system for examining the consequences of release from pathogen pressure for the purposes of a GM risk assessment, as the morphological characteristics and life history of *T. repens* are favourable for naturalisation of the transgenic, and/or gene flow and introgression of the transgene into naturalised *T. repens.* Recent

work conducted by Godfree *et al.* (2007) using *Clover yellow vein virus* (ClYVV) - *T. repens* pathosystem arrays demonstrated that ClYVV may reduce naturalised host population growth rates by 10% or higher when more than 50% of plants are infected. While this change appears relatively small, Godfree *et al.* (2007) demonstrated that under certain conditions ClYVV may drive otherwise viable naturalised host *T. repens* populations to extinction. In that situation, alterations in *T. repens* population growth rates were caused by reductions in a range of traits including stolon elongation rate, flower generation, survival and seed production. It is reasonable to expect similar results for AMV, which has been documented to cause significant reductions in cultivated *T. repens* performance (Campbell and Moyer, 1984, McLaughlin, 1992, Latch and Skipp, 1987). However, no published study has assessed the impact of AMV on naturalised *T. repens* populations.

The key objectives of the work presented in this chapter are to:- determine whether AMV isolates collected from naturalised *T. repens* populations reduce the growth and survival of local (from the same location), non-local (from a geographically separate location) and cultivated (agricultural cultivars) *T. repens* lines; and to infer the risk GM AMV-resistant *T. repens* may pose to non-target habitats in SE Australia. This process involves: i) determining the virulence and infectivity of SE Australian AMV isolates from naturalised *T. repens* populations; ii) determining the impacts of AMV on *T. repens* growth; and iii) consideration of the degree of environmental risk posed by AMV-resistant transgenic *T. repens* to non-target habitats in New South Wales (NSW), the Australian Capital Territory (ACT) and Victoria (Vic.).

In this study virus-free plants (a suitable non-GM substitute) are compared with clones that are infected with AMV in H-P arrays. Here, H-P arrays are designed to test the infectivity and growth impacts of AMV and to inform risk relevant to native habitats if the transgene is incorporated into the naturalised clover genome or the transgenic itself becomes naturalised, by testing the

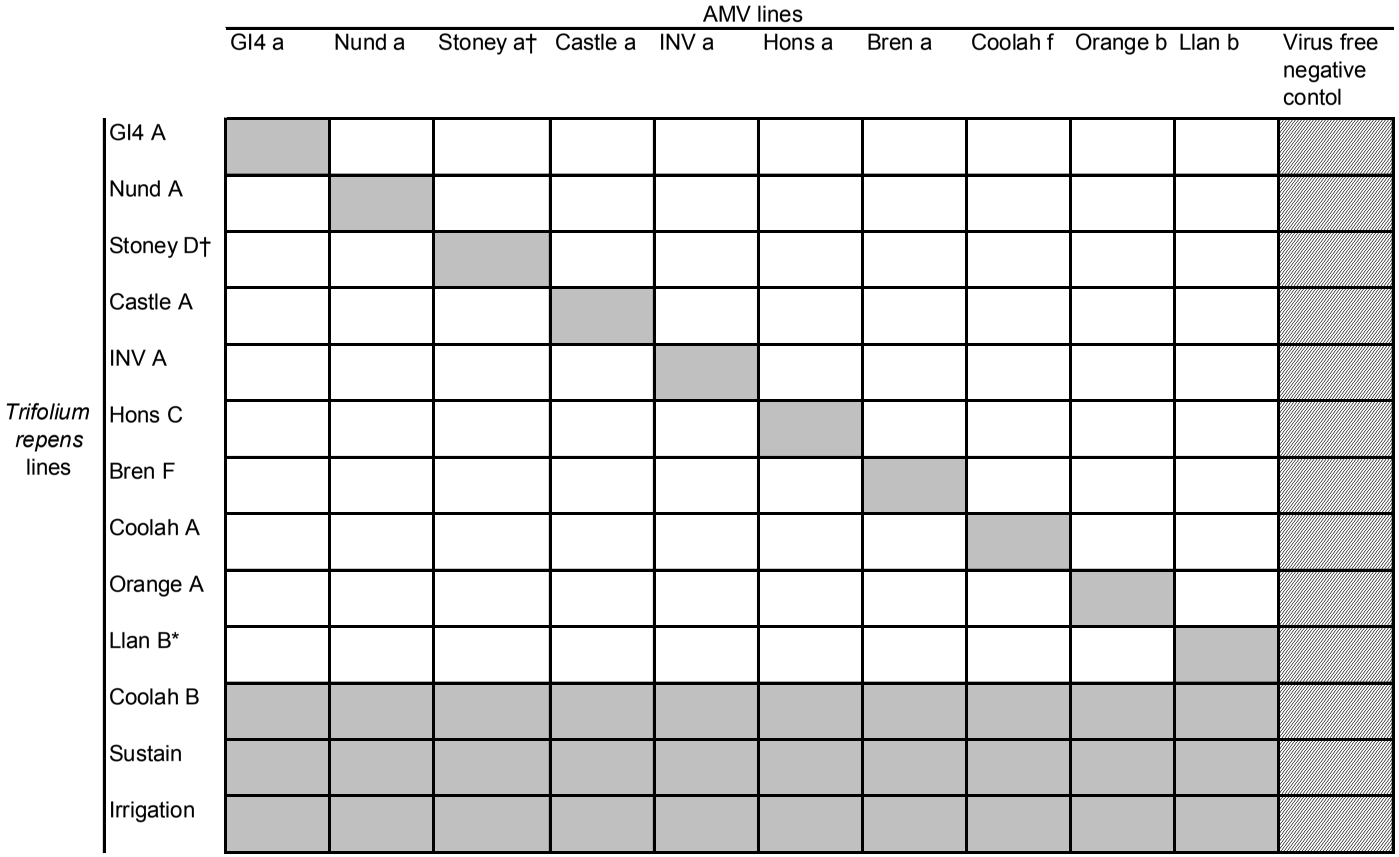
impacts of numerous AMV isolates on: i) local naturalised clover genotypes (reflecting the current status of H-P dynamics in naturalised systems); ii) cultivated clover genotypes (reflecting the possible H-P dynamics if the transgenic escapes); and iii) non-local *T. repens* (to detect diversity in the pathogenicity of AMV and alterations in the H-P dynamics that might occur when isolates are introduced into new areas). To determine if there is any diversity in the infectivity and virulence of AMV, clones of two commercial cultivars and one naturalised *T. repens* plant are challenged with AMV lines from 10 different geographic locations. Commercial *T. repens* lines are also examined because it is likely that GM AMV-resistant *T. repens* will be crossed with elite breeding lines prior to commercialisation. For that reason, knowledge of the susceptibility of these commercial cultivars to AMV is also useful for providing an indication of the potential yield gains that may result from the commercial production of GM AMV-resistant *T. repens.*

4.2 Methods

To quantify the infectivity of AMV and the impact of infection on the growth and survival of *Trifolium repens,* AMV-*T. repens* pathosystem arrays were established and the resultant plants were used in a glasshouse growth trial. Pathosystem arrays were designed to test the impact of AMV isolates on local clover (from the same site), non-local clover (from another site) and commercial clover lines. Two commercially available *T. repens* cultivars were selected and grown from seed: *Irrigation* and *Sustain*, along with naturalised virus-free plants from 10 sites in NSW: *GI4*, *INV1*, *Llan*, *Castle*, *Nundle*, *Coolah*, *Orange*, *Hons*, *Stoney* and *Bren* (site details are listed in Appendix 2.2). AMV isolates for the inoculation of experimental plants were selected from the same 10 sites from which virus-free naturalised *T. repens* were chosen (Figure 4.1 and Table 4.1).

Figure 4.1: Location of sites in NSW where wild *Trifolium repens* and AMV were collected for the inoculation and growth trials.

Table 4.1: Design of the inoculation trial. All *Trifolium repens* and AMV combinations for the trial are indicated by shaded rectangles. The inoculation trial contained a total of 53 treatments: Ten *Trifolium repens* lines inoculated with local AMV isolates; ten *Coolah B* clones each inoculated with a different AMV line; ten *Sustain* clones each inoculated with a different AMV line; and ten *Irrigation* clones each inoculated with a different AMV line. Virus free negative controls for all plant lines are indicated by striped rectangles.



*This clover was removed from the growth trial design as it was found to be co-infected with AMV and another virus.
†This clover-virus combination was removed from the growth trial design and included in a supplementary study (see methods).

4.2.1 *Trifolium repens* collections

Stolons of naturalised *T. repens* plants were collected between January 2006 and April 2007 as a part of a large survey conducted across NSW (described in Chapter 2) and stored in plastic bags on ice for transport. Within one week of collection, stolons were planted into 50 mm pots containing compost. Pots were kept covered with clear plastic in a growth room (20oC) for two weeks, and then transferred to a climate controlled glasshouse (day/night temperature 20oC) for further growth.

4.2.2 Virus detection

Collected *T. repens* plants to be used for the growth trial were tested for the presence of virus as described in Chapter 2. Briefly, three methods were used to detect viruses in collected *T. repens* and inoculated plants: i) indicator-plant bioassays for the detection of AMV, *Clover yellow vein virus* (ClYVV) and *White clover mosaic virus* (WClMV) were performed on indicator plants cowpea (*Vigna unguiculata)* and *Chenopodium amaranticolor* as described in Godfree *et al*. (2004b); ii) PCR for AMV detection (Chapter 2, Xu and Nie, 2006); and iii) immunoassays for the detection of AMV, ClYVV and WClMV (Graddon and Randles, 1986). The *T. repens* plants that were used as positive controls for AMV were collected from near Canberra, ACT. *Trifolium repens* grown from seed was used as a negative control for all tests since AMV is not seed transmitted in *T. repens* (Latch and Skipp, 1987).

4.2.3 Preparation of plants for inoculation trial

Clones of all chosen plants, including commercial cultivars, were prepared by taking cuttings 50 mm long from the stolons of virus free plants. Cuttings were planted in 50 mm diameter pots containing sterilised compost and grown in a growth cabinet at 20˚C with constant light.

4.2.4 Inoculation trial design

Ten naturalised *T. repens* plants and 10 virus isolates previously collected (Chapter 2) were used in conjunction with two commercially available *T. repens* cultivars: *Irrigation* and *Sustain*. AMV isolates and naturalised virus free plants were selected reflecting as diverse a range of non-target habitats as possible. The selected naturalised and commercial plants were clonally propagated and subjected to inoculation with the chosen 10 AMV lines (with appropriate negative controls).

Design of the AMV-*T. repens* inoculation array is illustrated in Table 4.1 and Figure 4.2. A total of 53 treatments, with two replicates for each treatment, were included in the inoculation trial. Treatments included: 10 naturalised *T. repens* lines inoculated with local AMV isolates (collected from the same site as the clover); 10 clones of a naturalised plant sourced from a site in the Central Tablelands NSW, *Coolah B*, inoculated with the 10 different AMV lines; 10 *Sustain* clones inoculated with the 10 AMV lines; 10 *Irrigation* clones inoculated with the 10 AMV lines. A virus-free negative control was included for each plant line (Table 4.1).

As illustrated in Figure 4.2, *T. repens,* collected from 10 different locations, infected with the selected AMV lines were used to inoculate *Nicotiana glutinosa* seedlings. This was done to isolate AMV, as many of the *T. repens* plants collected from the naturalised were also infected with ClYVV and WClMV (Chapter 2), and *N. glutinosa* is not a suitable host for these two viruses (Buchen-Osmond, 2002). Once *N. glutinosa* plants were symptomatic for AMV, they were tested by immunoassay (see virus detection) for the presence of AMV and absence of ClYVV and WClMV.

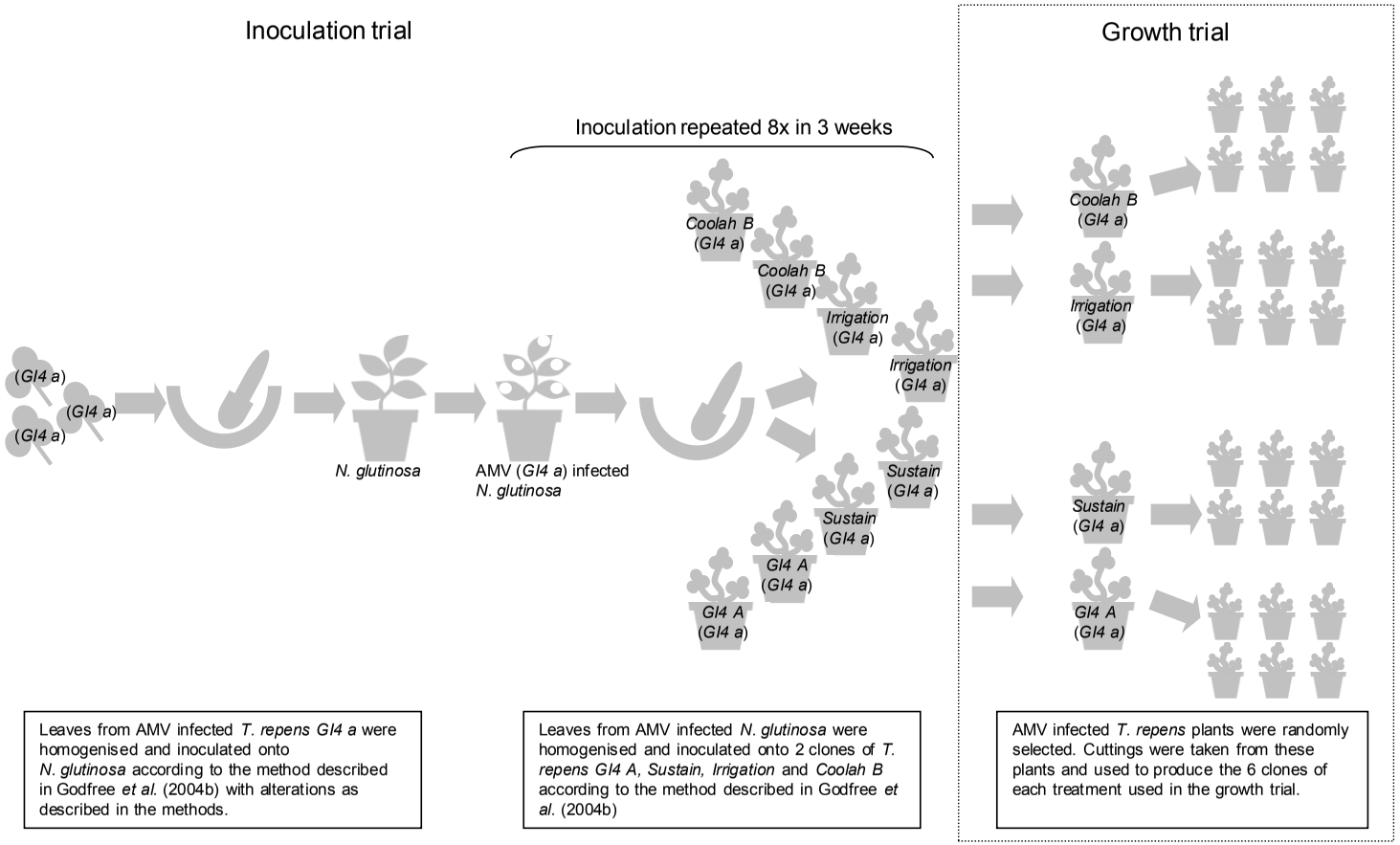


Figure 4.2: Flow diagram of the inoculation and growth trial. This figure illustrates how treatments were prepared using virus line *GI4 a* as an example. All treatment types are listed in Table 1. Appropriate negative controls for all treatments were produced in the same manner except they were inoculated with mixtures devoid of infected plant material.

4.2.5 Inoculation trial

Freshly propagated, less than one month old virus-free *T. repens* cuttings taken from naturalised and commercial *T. repens* (see preparation of plants for inoculation trial) were mechanically inoculated with leaf material from AMV-infected *N. glutinosa.* Inoculation was repeated eight times over three weeks in a temperature controlled cabinet at 20oC with constant light. Repeated inoculations were employed due to previous inoculation difficulties I had experienced. Barnett and Gibson (1975) also found some resistance in *T. repens* to mechanical inoculation with AMV.

Infected *N. glutinosa* leaf material was used for mechanical inoculation of *T. repens* stolons according to the bioassay method described by Godfree *et al.* (2004b) with some methodological changes (described below). In all cases as much leaf material as possible of the new host plant was inoculated. Negative controls were inoculated with mixtures devoid of virus infected plant material. Inoculated *T. repens* cuttings were tested for virus four and eight weeks after the first inoculation, with immunoassay and bioassay respectively (see virus detection). Initially the inoculation of AMV line *Stoney a* from *N. glutinosa* to *T. repens* failed, therefore the *T. repens* plants were inoculated an additional eight times over an additional three weeks. During immunoassay testing at four weeks one of the local *T. repens* lines, *Llan B*, was found to be infected with ClYVV and was subsequently removed from the growth trial design.

4.2.6 Growth trial design

A glasshouse trial was chosen as fitness costs tend to be more obvious in a controlled environment and may be more difficult to detect under field conditions (Parker and Kareiva, 1996). From the negative controls, and the plants successfully infected in the inoculation trial, one replicate of each treatment was randomly selected, cloned and organised into a growth trial in a random complete block design with six blocks (one replicate of each treatment per block). The AMV line *Stoney a* and clover line *Stoney D* were not included

in the growth trial as this clover line proved difficult to mechanically inoculate with *Stoney a* during the inoculation trial and extra attempts to inoculate were required. Once these were successfully inoculated they were subsequently incorporated into a supplementary study with relevant negative controls. Clover line *Bren F* inoculated with *Bren a*, with relevant negative controls, was also included in the supplementary study to test for the repeatability of results. The supplementary study lagged one month behind the main growth trial.

4.2.7 Growth trial

Pairs of AMV positive and AMV virus-free genetically identical *T. repens* clones randomly selected from the successfully infected inoculation array replicates, consisting of the four clover treatment groups *Sustain*, *Irrigation*, *Coolah* and nine local lines, were prepared for the growth trial (as illustrated in Figure 4.2). Six replicates of each treatment were prepared by taking cuttings 50 mm in length from the selected plants, potted into 50 mm pots with compost and grown in a cabinet at 20°C with constant light for three weeks to establish roots. The cuttings were acclimatised for one week in the glasshouse, (day/night temperature 20°C) and then allocated to six random complete blocks, with one of each replicate per block, following re-potting in 150 mm pots containing 50% compost and 50% sand. Symptoms of AMV infected plants can be masked at high temperatures (Graydon and Chu, 1993). As the strongest symptoms develop at 18-24 oC (Kreitlow and Price, 1949), the growth trial was conducted within this temperature range which also similar to that used in other published trials (e.g. Gibson *el al.* (1981)).

Plants were randomised within each block. The overall treatment design was the same as the inoculation trial (Table 4.1); except that the local *T. repens* lines *Llan B* and *Stoney D*, and virus line *Stoney a* were removed. The trial was conducted for two months with three census dates, when the plants were one month old (at the beginning of the trial), two months old and three months old (at the end of the trial). During the growth trial all plants were watered daily

and fertilised weekly with Hortico Aquasol, a water soluble fertiliser to ensure optimum growth. The growth trial was performed for three months because Godfree *et al.* (2009a) observed that for another viral clover pathogen, ClYVV, the largest impacts on growth and survival occurred in the first two months of growth, before root growth became restricted in pots. Plants were harvested at the final census.

Dry weight (yield) is the only growth trait measured in many growth studies of *T. repens* (Gibson *et al.*, 1982, Campbell and Moyer, 1984, Taylor *et al.*, 1995, Miller, 1962). However, in order to gain a greater understanding of AMV impacts on traits important for clover growth, morphology and competition, were measured in this study. The plant characteristics that were chosen to be measured in this growth trial had been measured in previous experiments assessing the performance of *T. repens* (Weijchedé *et al.*, 2008, Gibson *et al.*, 1981, Bouton *et al.*, 2005, Mclaughlin, 1996, Brink *et al.*, 1999, Lee *et al.*, 1993, Lane *et al.*, 2000). Plant growth variables were measured at each census included: 1) Plant height (mm); 2) width and height (mm) of up to five intact mature flower heads (Figure 4.3c); 3) number of flower heads; 4) the developmental stage of flower heads (immature, developing or mature) (Figure 4.3); 5) length of the longest stolon (mm); 6) leaflet width and length (mm) measured on the middle leaflet of the third or fourth fully expanded leaf from the end of the longest stolon; 7) internode length (mm) measured 50 mm from the end of the longest stolon; 8) internode thickness (mm) measured approximately 50 mm from the end of the longest stolon (between nodes); 9) number of branches on the longest stolon (only branches that resulted in secondary stolons ≥20 mm were counted); 10) number of primary stolons ≥20 mm; and 11) number of leaves (census one and two only). At harvest, roots were washed and separated from above ground material, placed in separate paper bags, dried at 50˚C until reaching constant weight (over three days) and weighed.

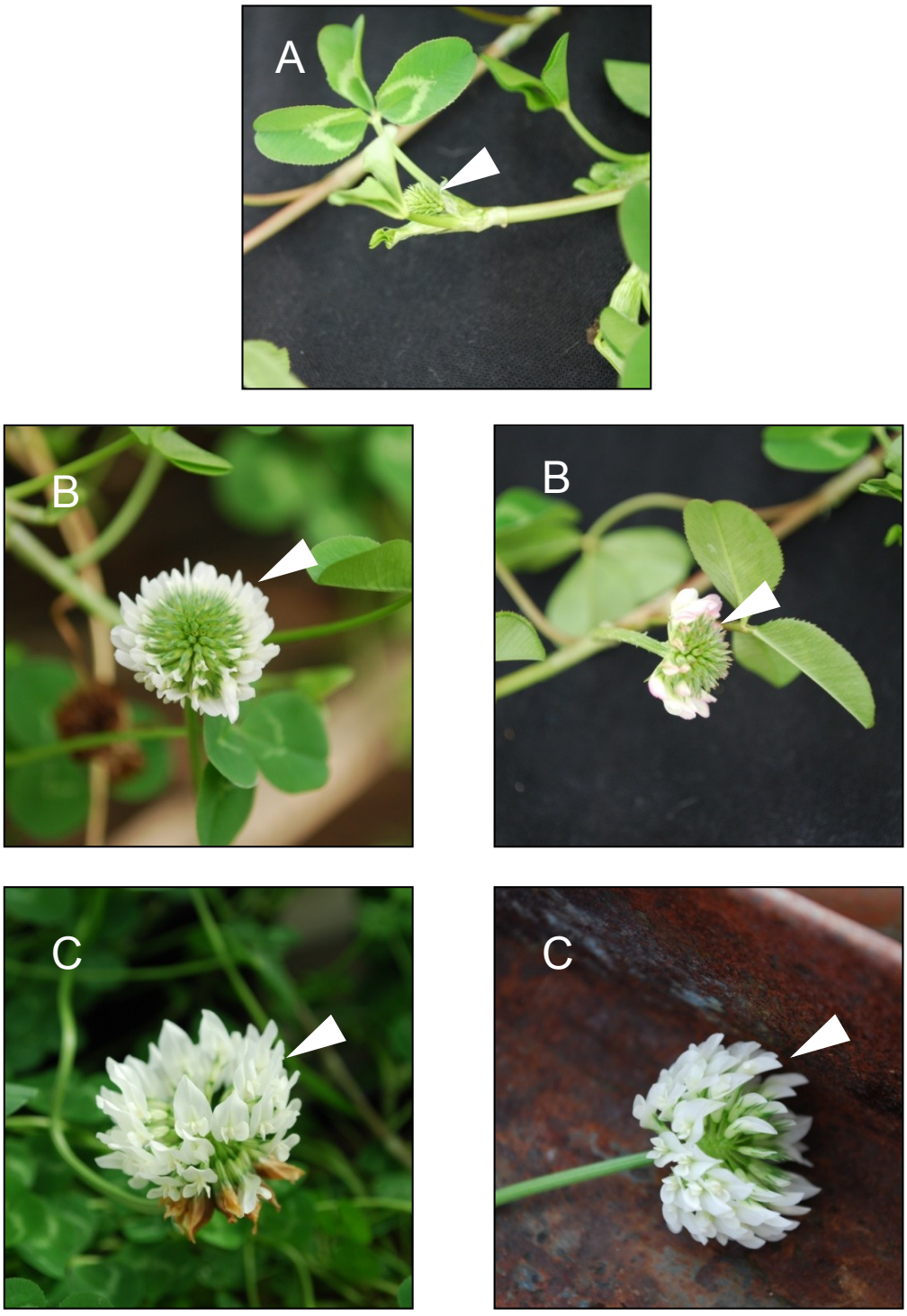


Figure 4.3: Stages of *Trifolium repens* inflorescence development. (A) depicts an immature inflorescence, (B) two examples of developing inflorescence and (C) mature inflorescence.

4.2.8 Statistical analyses

All statistical tests were performed in GenStat 12th edition (VSN International, 2009). Variables were transformed as necessary to improve the model assumptions and extreme outliers were removed prior to analysis. Clover line and AMV presence (V+/V-) were used as predictors of plant growth parameters with "block" as the random effect.

Growth results were analysed using analysis of variance (ANOVA) for balanced data sets, linear mixed model analysis for unbalanced data sets and data sets with missing data, and general linear mixed model analysis (Poisson distribution, logarithm link function) for count data such as number of flower heads, number of branches on the longest stolon and number of primary stolons. Data was analysed over time using repeated measures ANOVA. The means, standard errors and effects of clover genotype, virus line and the two-way interaction term were determined for each variable measured. The means, standard errors and least-square means were based on untransformed data.

The percentage relative virus effect (RVE), a measure of the reduction or increase of a growth parameter in response to AMV infection when virus free (V-) and virus positive (V+) plants were compared, was calculated as the percentage RVE= [(V+/V-)-1)*100] (Godfree *et al.*, 2009a). Contrasts of V+ and V- means were performed for all variables. V-/V+ contrasts were also used to compare means of specific virus x clover combinations. Dunnett's tests (90% and 95% confidence intervals) were utilised for unplanned post-hoc analysis of the variables "above ground dry weight" and "total dry weight" of clover lines *Sustain*, *Irrigation* and *Coolah B*.

4.3 Results

4.3.1 Inoculation trial

AMV inoculations resulted in infection of all *T. repens* lines except for the *Stoney* AMV line, which failed to infect clover lines *Coolah B* and *Sustain* (Table

4.2). Overall, 91% of inoculated plants were successfully infected. AMV line *Stoney a* infected only 25% of the *T. repens* plants inoculated, despite a repeat of the multiple inoculation procedure described in the methods. Commercial clover cultivars and naturalised genotypes exhibited similar infection rates (85-95%). One of the naturalised *T. repens* clones from the site Honeysuckle, NSW (*Hons C*) died during the inoculation trial due to unknown causes.

4.3.2 Growth trial

4.3.2.1 Visible effects of *Alfalfa mosaic virus* infection

All *T. repens* plants survived the growth trial and supplementary trial. The majority of plants remained visually healthy despite AMV infection. The most severe symptoms of AMV infection of *T. repens* observed during the growth trial included crinkling of the leaves, yellow blotching and streaking (Figure 4.4). However, in the majority of cases there was very little visible difference between AMV infected plants and their respective virus free clone (Figure 4.5).

4.3.2.2 Naturalised *Trifolium repens* inoculated with local *Alfalfa mosaic virus*

Analysis of variance of variables measured only at census 3 (C3) indicated that the clover x virus interaction was significant for above ground dry weight (AgDryWt) ($P = 0.017$) and mean total dry weight (Totwt) ($P = 0.016$), indicating that for these variables the main effects of clover line and virus depend in the specific virus-clover combination. A significant model effect of clover line was observed for root to shoot ratio (Rsratio) ($P = 0.018$) and a significant main effect of virus was observed for mean root dry weight [(Rootwt) ($P = 0.011$); relative virus effect (RVE) -20.8% (Table 4.3)]. Overall, when AMV infected and virus free plants were compared using contrasts, significant impacts were observed for the variables:- AgDryWt (RVE = -16.4% $P < 0.001$); Rootwt (RVE = -20.8% $P = 0.011$); and Totwt (RVE = -18.7% $P < 0.001$). However, as indicated by the model effect results, the RVE of growth parameters for individual clover

Table 4.2: Infectivity of AMV on *Trifolium repens* cuttings. *Trifolium repens* genotypes are divided into naturalised and commercial cultivars *Sustain* and *Irrigation*. Naturalised genotypes include local (from the same site as the respective virus source) and *Coolah B* (a genotype from the site "Coolah 1"). Data represent number of plants developing AMV infection/total number of clones inoculated.

			***Trifolium repens* genotype**				
			Naturalised		**Commercial**		
Virus source	**Region (NSW)**	**AMV line**	**Local**	**Coolah B**	**Sustain**	**Irrigation**	**TOTAL**
Stoney Creek	Southern Tablelands	Stoney a	1/2	0/2	0/2	1/2	2/8
Brennan's travelling stock reserve	South Coast and Illawarra	Bren a	2/2	2/2	2/2	2/2	8/8
Castletop	North West Slopes and Plains	Castle a	2/2	2/2	2/2	2/2	8/8
Inverell 1	North West Slopes and Plains	INV a	2/2	2/2	2/2	2/2	8/8
Glen Innes 4	Northern Tablelands	GI4 a	2/2	2/2	2/2	2/2	8/8
Little Llangothlin Reserve	Northern Tablelands	Llan b	2/2	2/2	2/2	2/2	8/8
Coolah 1	Central Tablelands	Coolah f	2/2	2/2	2/2	2/2	8/8
Orange	Central Tablelands	Orange b	2/2	2/2	2/2	2/2	8/8
Nundle	North West Slopes and Plains	Nund a	2/2	1/2	2/2	2/2	7/8
Honeysuckle	Southern Tablelands	Hons a	1/1*	2/2	2/2	2/2	7/7
TOTAL			**18/19**	**17/20**	**18/20**	**19/20**	**72/79 (91 %)**

*One cutting died

Figure 4.4: Symptoms of AMV infection of *Trifolium repens.* (A) depicts virus-free *Trifolium repens* and (B) the most severe symptoms of AMV infection observed during the study. Symptoms included crinkling, yellow streaks and blotches on the leaves.

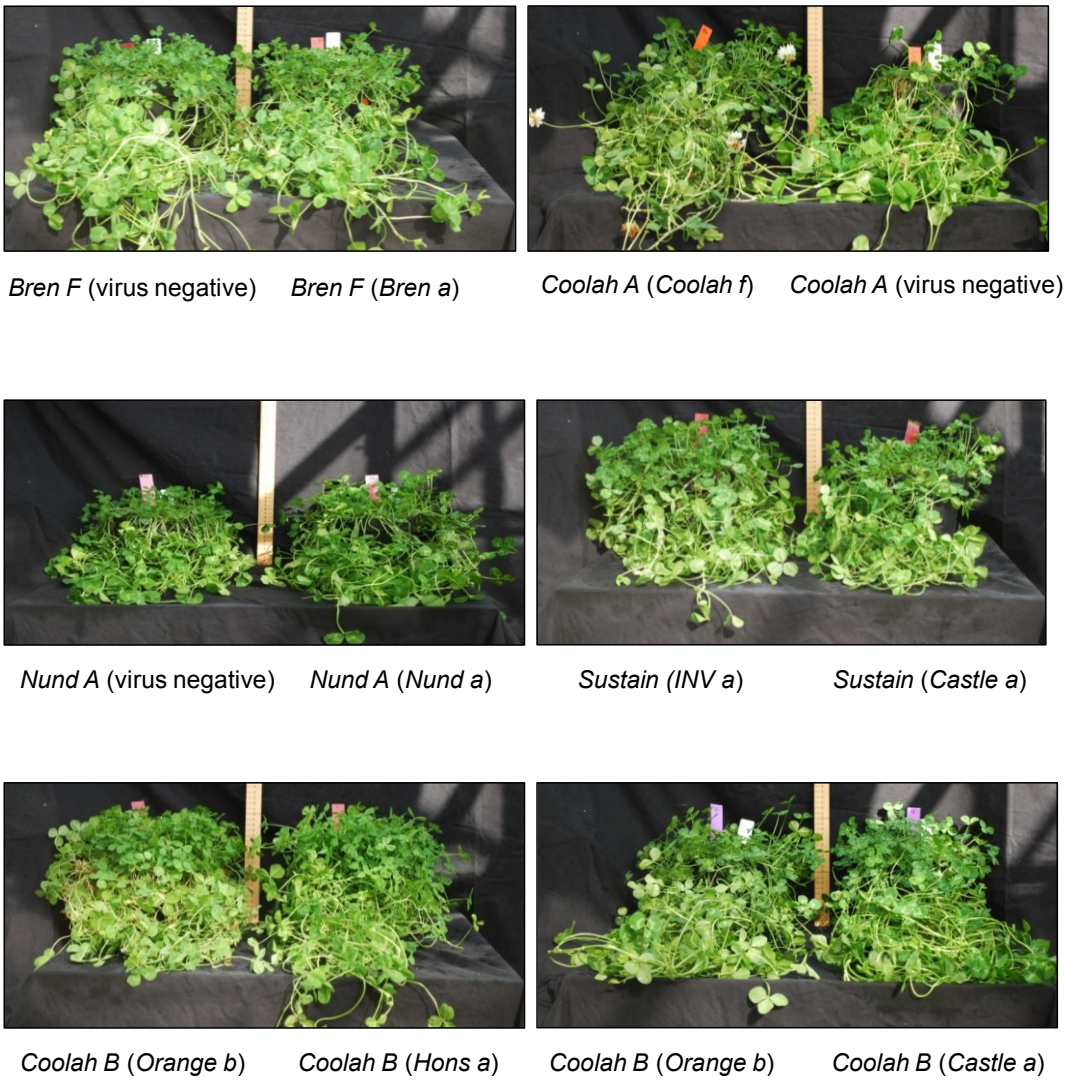


Figure 4.5: Images of a small sample of *Trifolium repens* plants from experiment 1 of the growth trial. *Trifolium repens* line (infecting AMV virus line). Pairs of plants originate from the same random complete block.

Table 4.3: Analysis of variance of naturalised clover infected with local AMV. The mean above ground dry weight (AgDryWt), root dry weight (Rootwt), total dry weight (TotWt) and root to shoot ratio (Rsratio) as measured after 3 months of growth. The model R^2 and model effects (clover (C), virus (V) and clover x virus (C x V) are presented. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are presented with the percentage relative virus effect (RVE=(V+/V-)-1)*100).

		Model effect			Means			
Variable	Model R^2	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*
AgDryWt	0.54	0.056	<0.001	0.017	26.27	21.97	-16.37	<0.001
Rootwt†	0.39	0.141	0.011	0.797	5.25	4.16	-20.76	0.011
TotWt	0.49	0.157	<0.001	0.016	32.14	26.12	-18.73	<0.001
Rsratio†	0.32	0.018	0.465	0.838	0.20	0.19	-4.99	0.217

†One outlier removed for analysis

Table 4.4: Repeated measures analysis of variance of naturalised clover infected with local AMV. The mean stolon thickness (StThick), length of the longest stolon (StolL), leaf length (Lfleng), leaf width (Lfwid), maximum plant height (Maxht) and internode length (Inleng) measured after one (census 1), two (census 2) and three (census 3) months of growth. The mean leaf number (Lvs) was only measured at census 1 and 2. The model R^2 and model effects (clover (C), virus (V), time (T), clover x virus (C x V), clover x time (C x T), virus x time (V x T) and clover x virus x time (C x V x T)) are presented. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are presented with the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each census.

									Means									
		Model effect							Census 1				Census 2				Census 3			
Variable	Model R^2	C	V	T	C x V	C x T	V x T	C x V x T	V-	V+	RVE (%)	V+/V- Contrast *P*	V-	V+	RVE (%)	V+/V- Contrast *P*	V-	V+	RVE (%)	V+/V- Contrast *P*
StThick	0.797	0.004	0.355	<0.001	0.295	0.002	0.286	0.319	2.21	2.17	-1.86	0.395	2.24	2.35	4.68	0.016	2.31	2.44	5.62	0.317
StolL†	0.959	<0.001	0.061	<0.001	0.730	0.008	0.466	0.475	134.60	126.30	-6.17	0.334	468.90	414.60	-11.58	0.015	614.90	552.90	-10.08	0.052
Lfleng	0.763	<0.001	0.069	<0.001	0.934	0.001	0.191	0.943	20.07	19.56	-2.54	0.443	24.58	23.83	-3.05	0.238	25.01	24.14	-3.48	0.103
Lfwid	0.837	<0.001	0.066	<0.001	0.242	0.005	0.956	0.079	16.54	15.84	-4.23	0.122	22.48	21.58	-4.00	0.146	23.07	22.16	-3.94	0.229
Lvs	0.932	0.054	<0.001	<0.001	0.288	0.078	0.001*	0.451	22.90	20.00	-12.66	0.026	149.80	123.00	-17.89	<0.001	N/A	N/A	N/A	N/A
Maxht	0.657	0.010	0.320	<0.001	0.713	0.030	0.011*	0.559	96.30	89.90	-6.65	0.196	100.10	111.80	11.69	0.014	107.50	112.70	4.84	0.297
Inleng	0.903	<0.001	0.027	<0.001	0.930	<0.001	0.136	0.382	26.07	23.26	-10.78	0.096	37.86	34.44	-9.03	0.057	35.11	31.90	-9.14	0.018

†Log(e) transformed for analysis

*Significant differences (*P* <0.05) in the V x T interaction were observed for Lvs between census 1 and 2, and census 2 and 3; and Maxht between census 1 and 2, and census 1 and 3.

lines were highly variable. Significant impacts were only observed in two clover x virus combinations for AgDryWt (*Bren F* x *Bren a* RVE = -43.4% *P* = 0.002 and *GI4 A* x *GI4 a* -34.2% *P* = 0.047), one for Rootwt (*Bren F* x *Bren a* RVE = -50.9% *P* = 0.05), and one combination for Totwt (*Bren F* x *Bren a* RVE = -51.6% *P* = 0.009) (Figure 4.6). When analysed by general linear mixed model (Poisson distribution, logarithm link function) the mean total inflorescence number (Totflow), the sum of the number of the inflorescence number observed at each census date, had a deviance of 188.03 (*P* <0.001), 85 degrees of freedom and differed significantly for the range of clover genotypes (*P* = 0.022). No significant difference was observed in the clover x virus interaction (*P* = 0.717) or the effect of AMV (*P* = 0.683) on flower number among clover lines for naturalised clover infected with local AMV.

For growth parameters measured across all census dates analysed by repeated measures analysis of variance [mean stolon thickness (StThick), mean length of longest stolon (StolL), mean leaf length (Lfleng), mean leaf width (Lfwid), mean maximum plant height (Maxht), mean internode length (Inleng) and mean leaf number (Lvs) (C1 and C2 only)], time is the most important factor (time main effect *P* < 0.001 for all variables), along with clover x time interactions (*P* range from 0.078 to < 0.001), indicating growth rate differences among clover genotypes (Table 4.4 and Appendix 4.1). The virus x time interaction is important for Lvs (*P* = 0.001) and Maxht (*P* = 0.011), indicating the need for census specific analysis. In addition, significant differences (*P* < 0.05) in the virus x time interaction were observed for StThick between C1 and C2, and C1 and C3; Lvs between C1 and C2, and C2 and C3; and Maxht between C1 and C2, and C1 and C3. The effect of virus infection is clearly important for Inleng (*P* = 0.027) and marginal for Lfwid (*P* = 0.066), Lfleng (*P* = 0.069) and StolL (*P* = 0.061). However, overall differences among clover lines exceeded that of virus effects, with clover line significant for all variables except Lvs, which was marginal (*P* = 0.054) (Table 4.4).

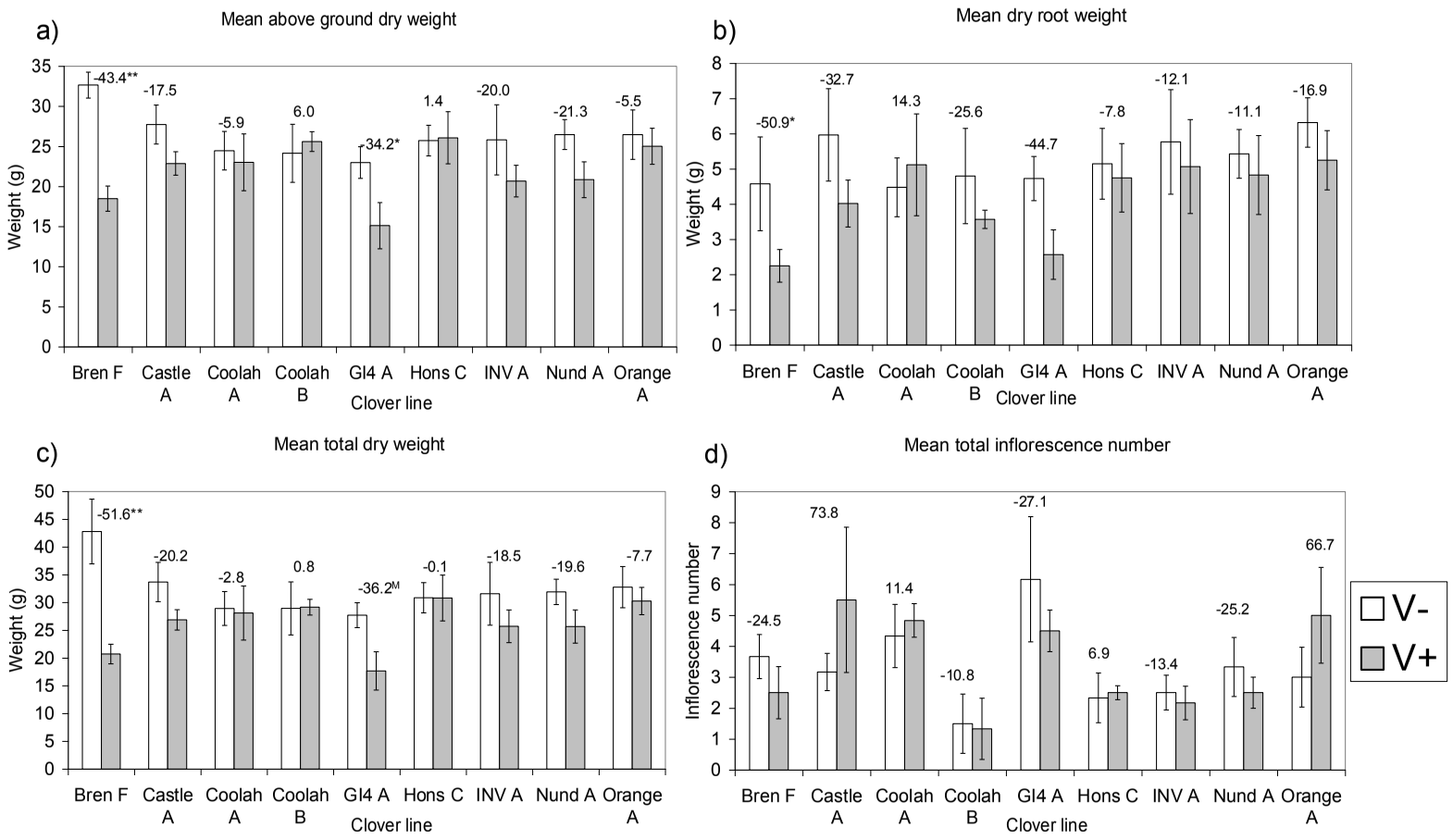


Figure 4.6: Naturalised clover inoculated with local AMV. Mean above ground dry weight a), dry root weight b) and total dry weight c) measured at census 3 (after 3 months growth). The mean total inflorescence number d) presented is a sum of the inflorescence number observed at each census date (1, 2 and 3 months). White bars represent the mean value of virus negative (V-) clover lines, grey bars represent the mean value of clover lines infected with local AMV (V+) and standard error of the mean is represented by error bars. The percentage relative virus effect (RVE= ((V+/V-)-1)*100) for each clover line is the value presented above V- and V+ pairs of bars. Significant V-/V+ contrasts are indicated by * (*P* = 0.01-0.05), ** (*P* = 0.001-0.01) or *** (*P* <0.001). Contrasts with marginal *P* values (0.05-0.1) are indicated by M.

Contrasts of V+/V- groups indicated significant or marginal impacts of AMV on:- StThick at C2 (RVE = +4.7% $P = 0.016$); StolL at C2 (RVE = 11.6% $P = 0.015$) and C3 (RVE = -10.1% $P = 0.052$); Lvs at C1 (RVE = -12.7% $P = 0.026$) and C2 (RVE = -17.9% $P < 0.001$); and Inleng at C1 (RVE = -10.8% $P = 0.096$), C2 (RVE = -9.0% $P = 0.057$) and C3 (RVE = -9.1% $P = 0.018$) (Table 4.4). The mean RVE across all virus-clover combinations for these variables and census dates was highly variable (Appendix 4.1 and 4.2). AMV had a consistently negative direction of impact at all census dates for variables: Inleng (RVE = -10.8 to -9.0% across census dates), Lfwid (RVE = -4.2 to -3.9), Lfleng (RVE = -3.5 to -2.5%) and StolL (RVE = -11.5 to -6.2%) (Table 4.4).

Variables that were analysed using repeated measures ANOVA (Table 4.4), that had significant model effects of interest, were re-analysed at individual census dates (Table 4.5). No significant differences were observed in the virus x clover interaction for any census dates for these growth parameters (Lvs was marginal at C1; $P = 0.06$). On average, the impact of AMV was significant for:- Lvs at C1 and C2 ($P = 0.026$ and $P < 0.001$ respectively); Maxht at C2 ($P = 0.014$); and Inleng at C2 and C3 ($P = 0.057$ and $P = 0.018$ respectively) [and marginal at C1 ($P = 0.096$)].

Results from analysis of count data collected at all census dates by general linear mixed model analysis (Poisson distribution, logarithm link function) were variable (Table 4.6). Significant differences were observed in the clover x virus interaction for:- the mean number of branches on the longest stolon (BranchNo) at C3 ($P = 0.022$); mean number of primary stolons (PriStNo) at C3 ($P = 0.019$) [and marginal at C2 ($P = 0.079$)]; and average inflorescence width (InfWid) at C1 ($P = 0.036$) [and marginal for mean average inflorescence height (InfHt) ($P = 0.052$)]. On average the mean inflorescence number (FHtot) of naturalised clover is unaffected by AMV infection as virus model effects for FHtot were not significant at any census. The clover genotype is more likely to act as the source of variation in flower number for naturalised clover, as the

Table 4.5: Variables analysed using repeated measures analysis of variance that had significant model effects of interest (Table 4.4) from the growth trial: naturalised clover infected with local AMV were re-analysed by analysis of variance at separate census dates. The model R^2 and model effects (clover (C), virus (V), clover x virus (C x V)) are presented for the mean number of leaves (Lvs) measured at census 1 (1 month of growth) and census 2 (2 months of growth), results are shown for mean maximum plant height (Maxht) and internode length (Inleng) measured at census 1, 2 and 3. The means of the virus free plants (V-) and AMV infected plants (V+) and the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each variable are presented in Table 4.4. Data was not available (N\A) to calculate model effects at census 3 for Lvs.

	Census 1				Census 2				Census 3			
		Model Effect				Model Effect				Model Effect		
Variable	Model R^2	C	V	C x V	Model R^2	C	V	C x V	Model R^2	C	V	C x V
Lvs	0.398	0.004	0.026	0.060	0.388	0.053	<0.001	0.329	N\A	N\A	N\A	N\A
Maxht	0.203	0.302	0.196	0.635	0.333	0.009	0.014	0.379	0.366	0.003	0.297	0.783
Inleng	0.495	<0.001	0.096	0.243	0.386	<0.001	0.057	0.954	0.494	<0.001	0.018	0.178

Table 4.6: Naturalised clover infected with local AMV. All variables were measured after one (census 1), two (census 2) and three (census 3) months of growth. The mean number of branches on the longest stolon (BranchNo), number of primary stolons (PriStNo) and total inflorescence number (FHTot) at each census was analysed by general linear mixed model analysis (Poisson distribution, logarithm link function). The mean average inflorescence height (InfHt) and average inflorescence width (InflWid) were evaluated by linear mixed model analysis. The model effects (clover (C), virus (V), clover x virus (C x V)) are presented for all variables at each census. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are presented with the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each census. Data was not available (N\A) to calculate model effects and contrasts at census 2 and 3 for InfHt and InfWid.

	Census 1							Census 2							Census 3						
	Model Effect			Means				Model Effect			Means				Model Effect			Means			
Variable	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*
BranchNo	0.378	0.983	0.452	0.63	0.69	8.83	0.743	<0.001	0.887	0.652	7.91	7.80	-1.40	0.880	0.027	0.216	0.022	11.04	9.83	-10.96	0.204
PriStNo	0.044	0.005	0.178	1.89	1.54	-18.63	0.006	0.198	<0.001	0.079	10.22	7.65	-25.18	<0.001	0.086	<0.001	0.019	26.27	21.97	-16.37	<0.001
FHTot	0.009	0.669	0.865	1.82	1.70	-6.12	0.646	0.025	0.958	0.573	1.13	1.13	0.00	1.000	0.001	0.118	0.668	0.39	0.59	52.38	0.455
InfHt	0.075	0.348	0.052	23.83	25.35	6.38	0.010	N\A	N\A	N\A	22.07	23.93	8.43	0.048	N\A	N\A	N\A	26.63	30.54	14.68	N\A
InfWid	0.222	0.388	0.036	26.39	25.56	-3.15	0.673	N\A	N\A	N\A	26.35	28.44	7.93	0.106	N\A	N\A	N\A	36.47	33.74	-7.49	N\A

clover line effect was significant for all census dates (*P* for C1= 0.009, C2 = 0.025 and C3 = 0.001). Contrasts indicated significant overall RVE of AMV on:- PriStNo at C1 (RVE = -18.6% *P* = 0.006), C2 (RVE = -25.2% *P* <0.001) and C3 (RVE = -16.4% *P* <0.001); and InfHt at C1 (RVE = +6.4% *P* = 0.01) and C2 (RVE = +8.4% *P* = 0.048). Results in Table 4.6 indicate that, for these variables, growth is generally dependent on the specific virus-clover genotype combination. This is illustrated graphically for BranchNo and PriStNo in Appendix 4.1h and i. The mean RVE across all virus-clover combinations of these two growth parameters was highly variable (see Appendix 4.1h and i), with significant or marginal results ranging from RVE = +18.6% *P* = 0.023 (BranchNo C3 *Bren F* x *Bren a*) to RVE = -65.4% *P* = 0.002 (PriStNo C3 *Bren F* x *Bren a*).

4.3.2.3 Commercial cultivars (*Irrigation* and *Sustain*) and one naturalised clover line (*Coolah B*) inoculated with 10 *Alfalfa mosaic virus* lines

No significant clover x virus interaction was detected for variables only measured at C3: AgDryWt, Rootwt, Rsratio, Totwt, or Tflow) (Table 4.7). While not all significant, the RVE for all variables was consistently negative. Virus effect was significant for AgDryWt (RVE = -13.6% *P* = 0.021) and marginal for Totwt (RVE = -13.8% *P* = 0.055). Clover line effects were highly significant (*P* < 0.001) for all variables (Table 4.7), indicating that differences among clover genotypes are large. Individual virus-clover combinations for variables are graphed in Figure 4.7. No significant V-/V+ contrast results were observed for clover line *Coolah B* for any of these variables, however a significant or marginal RVE was observed for *Irrigation* in AgDryWt (RVE = -16.3% *P* = 0.098) and *Sustain* in AgDryWt (RVE = -20.4% *P* = 0.023) and Totwt (RVE = -20.1% *P* = 0.025).

Results of repeated measures ANOVA across nine growth variables and all census dates is presented in Table 4.8 and Figure 4.8. A significant clover x virus x time interaction was only observed for Maxht (*P* = 0.003) [InfHt

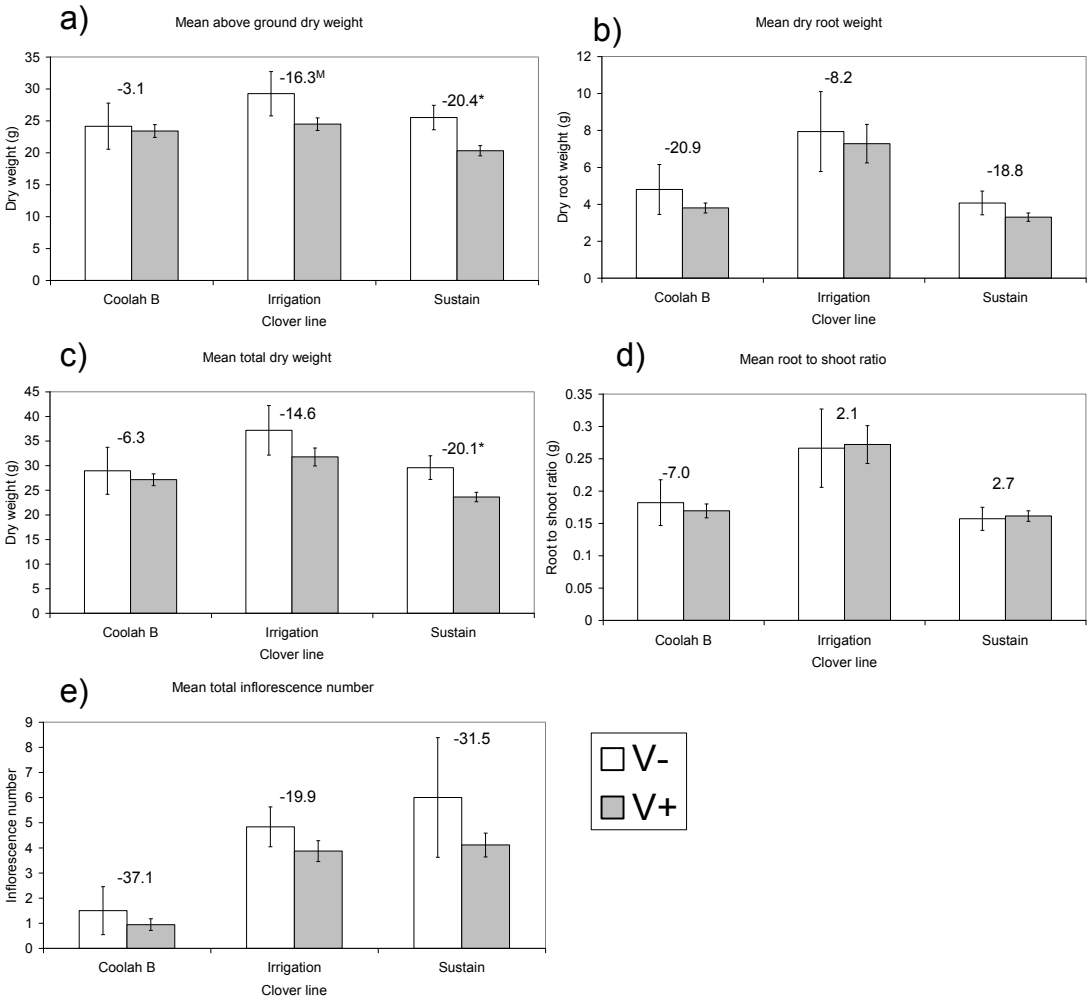


Figure 4.7: Two commercial (*Irrigation* and *Sustain*) and one naturalised clover line (*Coolah B*) infected with nine AMV lines. Mean above ground dry weight a), dry root weight b), total dry weight c) and root to shoot ratio d) measured at the final census date (after 3 months growth) are presented. The mean total inflorescence number e) presented is a sum of the inflorescence number observed at each census date (1, 2 and 3 months). White bars represent the mean value of virus negative (V-) clover lines, grey bars represent the mean value of clover lines infected with AMV (V+) and the standard error of the mean is represented by error bars. The percentage relative virus effect (RVE= ((V+/V-)-1)*100) for each clover line is presented above V- and V+ pairs of bars. Significant V-/V+ contrasts are indicated by * (*P* = 0.01-0.05) and contrasts with marginal *P* values (0.05-0.1) are indicated by M.

Table 4.7: Clones of one naturalised (*Coolah B*) and two commercial (*Sustain* and *Irrigation*) clovers infected with 10 AMV lines. The mean above ground dry weight (AgDryWt), root dry weight (Rootwt), root to shoot ratio (Rsratio) and total dry weight (Totwt) measured at the final census (after 3 months of growth) are presented. The mean total inflorescence number (Tflow) presented is a sum of the inflorescence number observed at each census date (1, 2 and 3 months). Data sets were analysed by linear mixed model analysis except Tflow which was analysed by general linear mixed model analysis (Poisson distribution, logarithm link function). The deviance, degrees of freedom (Df), the probability of the deviance (Pr. Dev) and model effects (clover (C), virus (V) and clover x virus (C x V)) are presented. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are shown with the percentage relative virus effect (RVE=(V+/V-)-1)*100).

				Model effect			Means			
Variable	Deviance	Df	Pr. Dev	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*
AgDryWt	825.14	172	<0.001	<0.001	0.020	0.452	26.31	22.74	-13.57	0.021
Rootwt	702.74	171	<0.001	<0.001	0.582	0.936	5.60	4.80	-14.27	0.467
Rsratio	-517.8	171	<0.001	<0.001	0.834	0.901	0.20	0.20	-0.45	0.990
Totwt	954.76	171	<0.001	<0.001	0.065	0.729	31.91	27.51	-13.79	0.055
Tflow	457.58	169	<0.001	<0.001	0.161	0.593	4.11	2.98	-27.63	0.120

Table 4.8: Repeated measures analysis of variance of clones of one naturalised (*Coolah B*) and two commercial (*Sustain* and *Irrigation*) clovers infected with 10 AMV lines. The mean internode length (Inleng), length of the longest stolon (StolL), stolon thickness (StThick), maximum plant height (Maxht), leaf length (Lfleng), leaf width (Lfwid), average inflorescence width (InfWid) and average inflorescence height (InfHt) measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth) are presented. The mean leaf number (Lvs) was only measured at census 1 and 2 and not enough data was available to provide results for InfWid at census 3. The model R^2 and model effects (clover (C), virus (V), time (T), clover x virus (C x V), clover x time (C x T), virus x time (V x T) and clover x virus x time (C x V x T)) are presented. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are presented with the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each census. Data was not available (N\A) to calculate model effects at census 3 for Lvs and InfWid.

		Model Effect							Census 1 Means				Census 2 Means				Census 3 Means			
Variable	Model R^2	C	V	T	C x V	C x T	V x T	C x V x T	V-	V+	RVE (%)	V+/V- Contrast *P*	V-	V+	RVE (%)	V+/V- Contrast *P*	V-	V+	RVE (%)	V+/V- Contrast *P*
Inleng	0.595	0.008	0.032	<0.001	0.446	0.114	0.005	0.243	33.93	25.49	-24.87	<0.001	35.51	36.76	3.52	0.508	34.43	32.28	-6.24	0.279
StolL	0.935	<0.001	0.360	<0.001	0.133	<0.001	0.787	0.182	166.90	137.50	-17.62	0.027	488.90	462.70	-5.36	0.403	627.40	611.50	-2.53	0.690
StThick	0.387	<0.001	0.233	0.219	0.839	0.644	0.981	0.943	2.18	2.33	6.69	0.672	2.26	2.41	6.45	0.010	2.32	2.48	6.72	0.038
Maxht	0.589	0.094	0.313	<0.001	0.584	0.507	0.869	0.003	101.56	94.75	-6.71	0.331	114.40	111.60	-2.45	0.609	119.70	115.40	-3.59	0.527
Lvs	0.906	<0.001	0.037	<0.001	0.558	0.004	0.120	0.867	22.94	18.57	-19.05	0.034	136.90	118.20	-13.66	0.050	N/A	N/A	N/A	N/A
Lfleng	0.637	0.197	0.356	<0.001	0.069	0.068	0.503	0.378	21.51	20.32	-5.53	0.177	24.41	24.50	0.37	0.912	25.76	25.08	-2.64	0.500
Lfwid	0.664	0.078	0.673	<0.001	0.410	0.799	0.670	0.367	16.74	16.47	-1.61	0.663	21.04	21.67	2.99	0.499	21.98	22.44	2.09	0.672
InfWid	0.859	0.013	0.028	<0.001	0.001	<0.001	0.020	0.226	29.76	26.01	-12.60	0.002	29.24	26.29	-10.09	0.530	N/A	N/A	N/A	N/A
InfHt	0.751	0.532	0.256	<0.001	0.102	0.004	0.105	0.088	27.43	24.67	-10.06	0.048	22.55	21.79	-3.37	0.081	29.76	26.01	-12.60	0.213

Figure 4.8

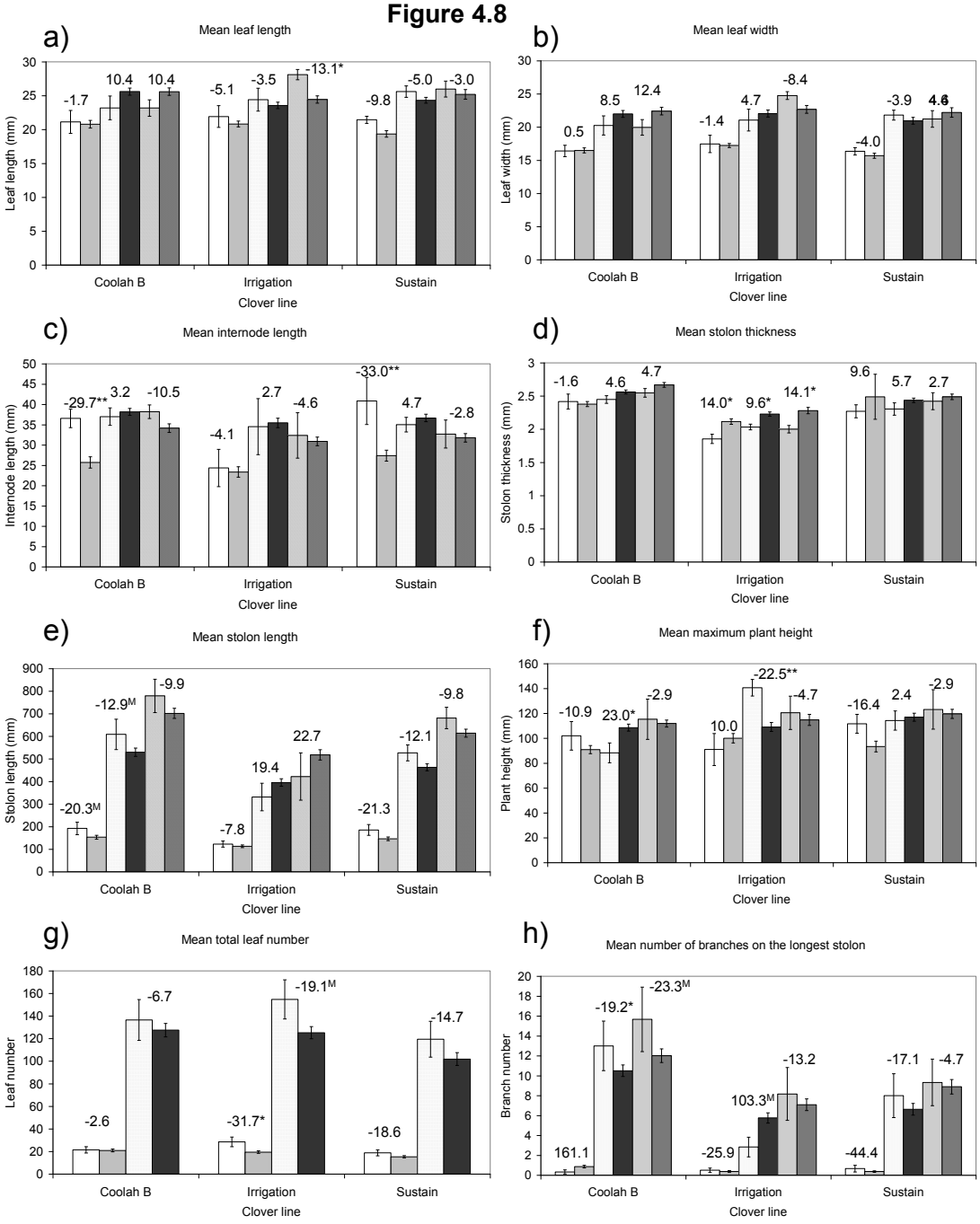

a)
Mean leaf length
Leaf length (mm)
-1.7
10.4
10.4
-5.1
-3.5
-13.1*
-9.8
-5.0
-3.0
Coolah B
Irrigation
Sustain
Clover line
b)
Mean leaf width
Leaf width (mm)
0.5
8.5
12.4
-1.4
4.7
-8.4
-4.0
-3.9
Coolah B
Irrigation
Sustain
Clover line
c)
Mean internode length
Internode length (mm)
-29.7**
3.2
-10.5
-4.1
2.7
-4.6
-33.0**
4.7
-2.8
Coolah B
Irrigation
Sustain
Clover line
d)
Mean stolon thickness
Stolon thickness (mm)
-1.6
4.6
4.7
14.0*
9.6*
14.1*
9.6
5.7
2.7
Coolah B
Irrigation
Sustain
Clover line
e)
Mean stolon length
Stolon length (mm)
-20.3M
-12.9M
-9.9
-7.8
19.4
22.7
-21.3
-12.1
-9.8
Coolah B
Irrigation
Sustain
Clover line
f)
Mean maximum plant height
Plant height (mm)
-10.9
23.0*
-2.9
10.0
-22.5**
-4.7
-16.4
2.4
-2.9
Coolah B
Irrigation
Sustain
Clover line
g)
Mean total leaf number
Leaf number
-2.6
-6.7
-31.7*
-19.1M
-18.6
-14.7
Coolah B
Irrigation
Sustain
Clover line
h)
Mean number of branches on the longest stolon
Branch number
161.1
-19.2*
-23.3M
-25.9
103.3M
-13.2
-44.4
-17.1
-4.7
Coolah B
Irrigation
Sustain
Clover line

Figure 4.8: continued

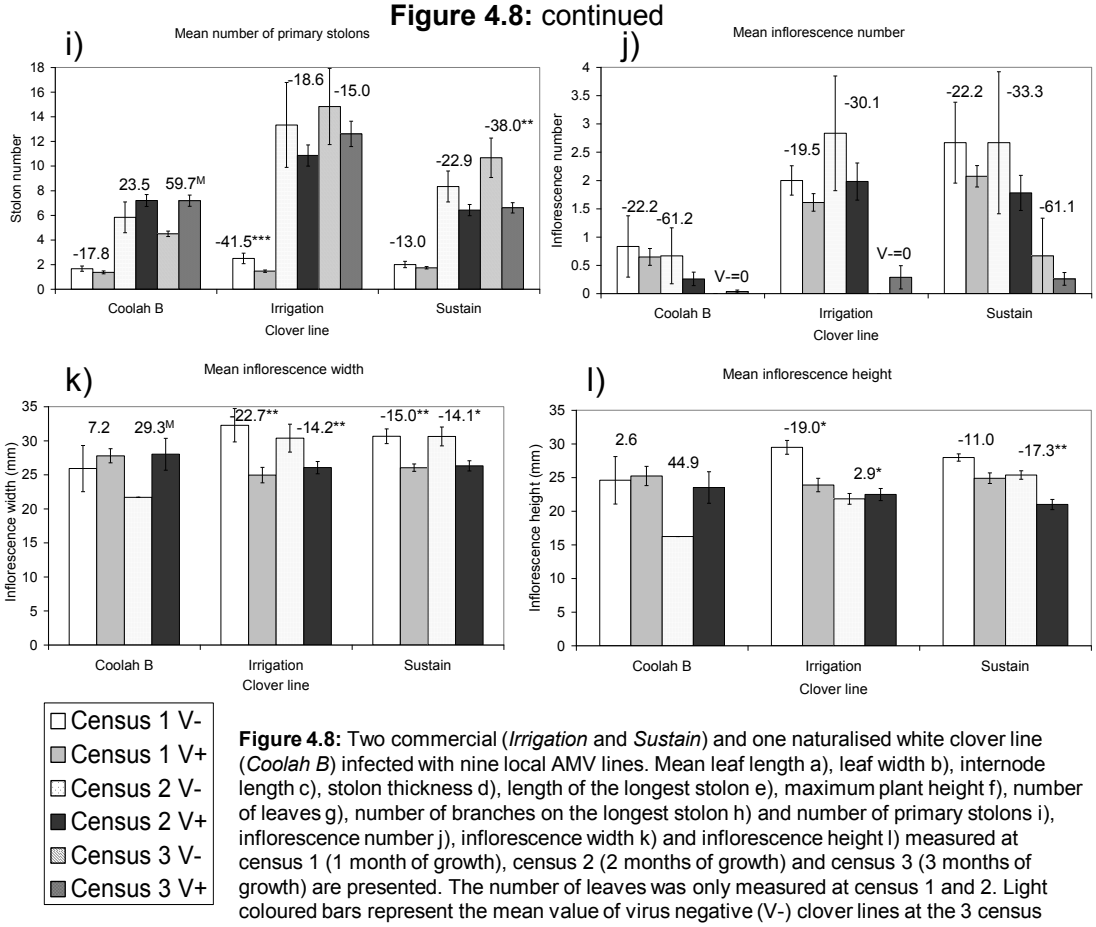


Figure 4.8: Two commercial (*Irrigation* and *Sustain*) and one naturalised white clover line (*Coolah B*) infected with nine local AMV lines. Mean leaf length a), leaf width b), internode length c), stolon thickness d), length of the longest stolon e), maximum plant height f), number of leaves g), number of branches on the longest stolon h) and number of primary stolons i), inflorescence number j), inflorescence width k) and inflorescence height l) measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth) are presented. The number of leaves was only measured at census 1 and 2. Light coloured bars represent the mean value of virus negative (V-) clover lines at the 3 census dates and shaded bars represent the mean value of clover lines infected with local AMV (V+) at the 3 census dates. The standard error of the mean is represented by error bars. The percentage relative virus effect (RVE= ((V+/V-)-1)*100) for each clover line is the value presented above each V+ and V- pair of bars for each census. Significant V-/V+ contrasts are indicated by * (*P* = 0.01-0.05), ** (*P* = 0.001-0.01) or *** (*P* <0.001). Contrasts with marginal *P* values (0.05-0.1) are indicated by M.

was marginal ($P = 0.088$)]. The clover x virus interaction was significant only for InfWid ($P = 001$) and marginal for Lfleng and InfHt ($P = 0.069$ and $P = 0.102$), indicating that the magnitude of these variables was dependent on the specific virus-clover combination. Clover x time and time model effects were generally significant for all the variables analysed, although to a lesser extent than for naturalised clover lines inoculated with local AMV, indicating that the growth characteristics of the clover lines *Irrigation, Sustain* and *Coolah B* changed over time and the genotypes grew at different rates. Virus effect was significant for Inleng ($P = 0.032$) and Lvs ($P = 0.037$), indicating that AMV influenced the growth of these variables. However, in some cases virus model effects were not significant and clover line was significant or marginal, indicating that differences among clover genotypes generally have more influence on growth than AMV infection for StolL ($P < 0.001$), StThick ($P < 0.001$) and Lfwid ($P = 0.078$). Overall significant or marginal results for V-/V+ contrasts were observed for InLeng at C1 (RVE = -24.9% <0.001); StolL at C1 (RVE = -17.6% $P = 0.027$); StThick at C2 (RVE = +6.5% $P = 0.01$) and C3 (RVE = +6.7% $P = 0.038$); Lvs at C1 (RVE = -19.1% $P = 0.034$) and at C2 (RVE = -13.7% $P = 0.05$); InfWid at C1 (RVE = -12.6% $P = 0.002$) and InfHt at C1 (RVE = -10.1% $P = 0.048$) and at C2 (RVE = -3.4% $P = 0.081$) (Table 4.8).

Variables analysed using repeated measures ANOVA (Table 4.8) that had significant model effects of interest were re-analysed at individual census dates (Table 4.9). Significant virus x clover interactions were observed for Lfleng at C3 ($P = 0.047$) and InfWid at C2 ($P = 0.033$). Virus x clover model effects were marginal for InLeng at C1 ($P = 0.093$), InfWid at C1 ($P = 0.109$) and Lvs at C2 ($P = 0.05$). Virus model effect was significant for Lvs at C1 ($P = 0.034$) and marginal at C2 ($P = 0.05$). Clover model effect was significant for Lfleng at C2 ($P = 0.025$) and InLeng at C3 ($P = 0.041$), and marginal for Lfleng at C1 ($P = 0.066$).

Table 4.9: Variables analysed using repeated measures analysis of variance (Table 4.8) that had significant model effects of interest from the growth trial: clones of one naturalised (*Coolah B*) and two commercial (*Sustain* and *Irrigation*) clovers infected with 10 AMV lines were re-analysed by linear mixed model analysis at separate census dates. The deviance, degrees of freedom (Df), the probability of the deviance (Pr. Dev) and model effects (clover (C), virus (V) and clover x virus (C x V)) are presented for the mean leaf length (Lfleng), internode length (InLeng) and average inflorescence width (InfWid) measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth). The mean leaf number (Lvs) was only measured at census 1 and 2 and not enough data was available to provide results for InfWid at census 3. The means of the virus free plants (V-) and AMV infected plants (V+) and the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each variable are presented in Table 4.8. Data was not available (N\A) for model effects to be calculated at census 3 for InfWid and Lvs.

	Census 1						Census 2						Census 3					
				Model Effect						Model Effect						Model Effect		
Variable	Deviance	Df	Pr. Dev	C	V	C x V	Deviance	Df	Pr. Dev	C	V	C x V	Deviance	Df	Pr. Dev	C	V	C x V
Lfleng	632.43	171	<0.001	0.066	0.178	0.718	624.50	172	<0.001	0.025	0.912	0.155	684.73	172	<0.001	0.717	0.500	0.047
InLeng	989.64	172	<0.001	0.014	<0.001	0.093	896.66	172	<0.001	0.152	0.508	0.987	912.92	172	<0.001	0.041	0.279	0.791
InfWid	317.37	77	<0.001	0.301	0.032	0.109	192.44	53	<0.001	0.964	0.024	0.033	N\A	N\A	N\A	N\A	N\A	N\A
Lvs	925.68	172	<0.001	<0.001	0.034	0.231	1468.79	172	<0.001	<0.001	0.050	0.676	N\A	N\A	N\A	N\A	N\A	N\A

Table 4.10: General linear mixed model analysis (Poisson distribution, logarithm link function) of clones of one naturalised (*Coolah B*) and two commercial (*Sustain* and *Irrigation*) clovers infected with 10 AMV lines. All variables were measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth). The mean number of branches on the longest stolon (BranchNo), number of primary stolons (PriStNo) and total inflorescence number (FHTot) at each census are shown. The model effects (clover (C), virus (V), clover x virus (C x V)) are presented. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are shown with the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each census.

	Census 1							Census 2							Census 3						
	Model Effect			Means				Model Effect			Means				Model Effect			Means			
Variable	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*
BranchNo	0.003	0.758	0.247	0.50	0.54	7.40	0.842	<0.001	0.52	0.08	7.94	7.63	-3.95	0.762	<0.001	0.17	0.76	11.06	9.33	-15.64	0.180
PriStNo	0.031	0.004	0.225	2.06	1.53	-25.83	0.004	<0.001	0.29	0.33	9.17	8.16	-10.99	0.363	<0.001	0.17	0.05	10.00	8.80	-11.98	0.343
FHTot	<0.001	0.244	0.997	1.83	1.44	-21.22	0.207	<0.001	0.15	0.85	2.06	1.34	-34.82	0.148	0.30	0.38	0.99	0.22	0.19	-12.78	0.910

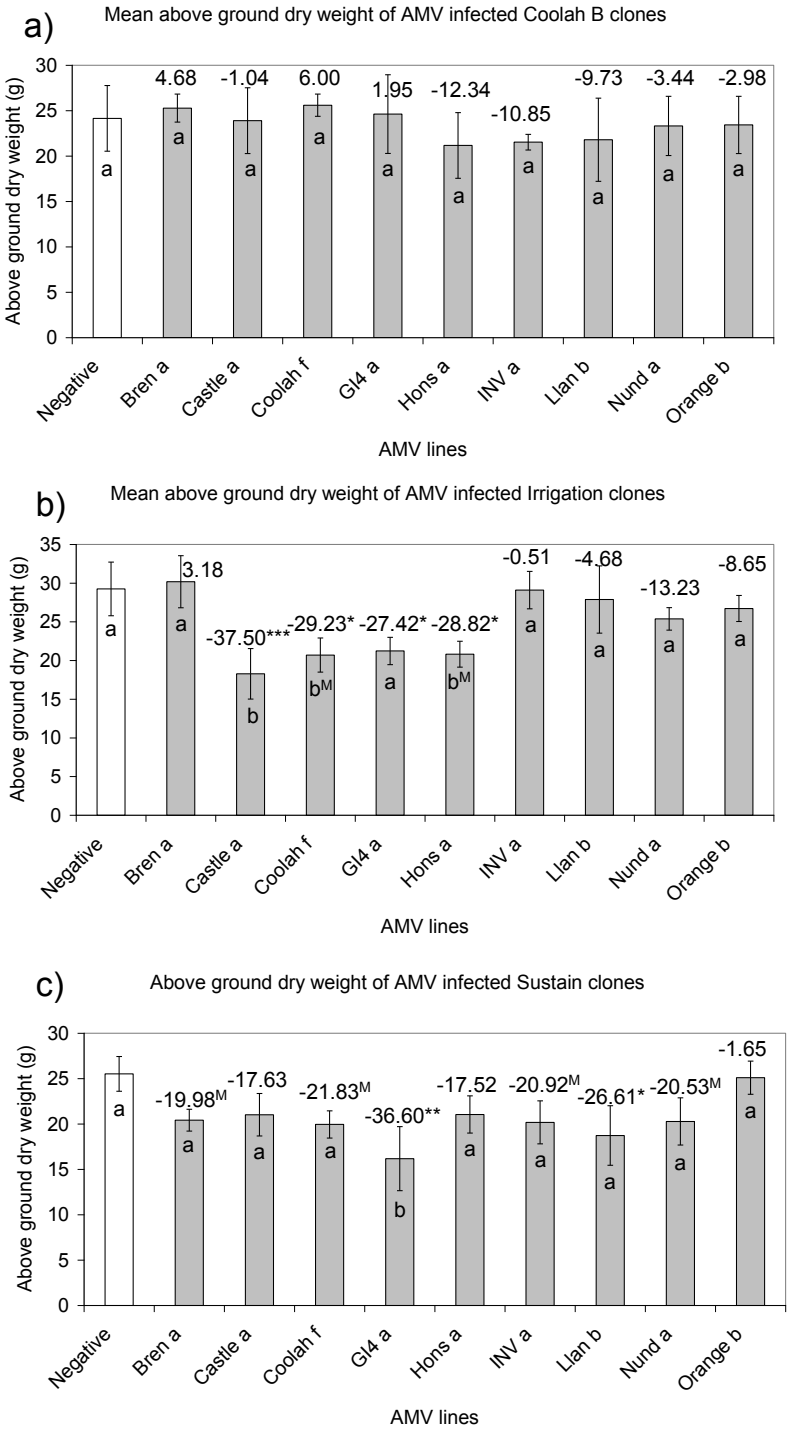


Figure 4.9: Two commercial (*Irrigation* and *Sustain*) and one naturalised white clover line (*Coolah B*) infected with nine AMV lines. Mean above ground dry weight of AMV infected *Coolah B* clones a), *Irrigation* clones b) and *Sustain* clones c) measured at census 3 (after 3 months of growth) is presented. White bars represent the mean value of virus negative (V-) clones and grey bars represent the mean value of clover lines infected with individual AMV lines (V+). The standard error of the mean is represented by error bars. The percentage relative virus effect (RVE= ((V+/V-)-1)*100) for each virus line is reported above each bar. Significant V-/V+ contrasts are indicated by * (*P* = 0.01-0.05), ** (*P* = 0.001-0.01) or *** (*P* <0.001). Contrasts with marginal *P* values (0.05-0.1) are indicated by M. Groups with Dunnett test (95% confidence interval) results significantly different from V- are identified by different letters. Groups with marginal Dunnett test results (90% confidence interval) are identified by a different letter followed by M.

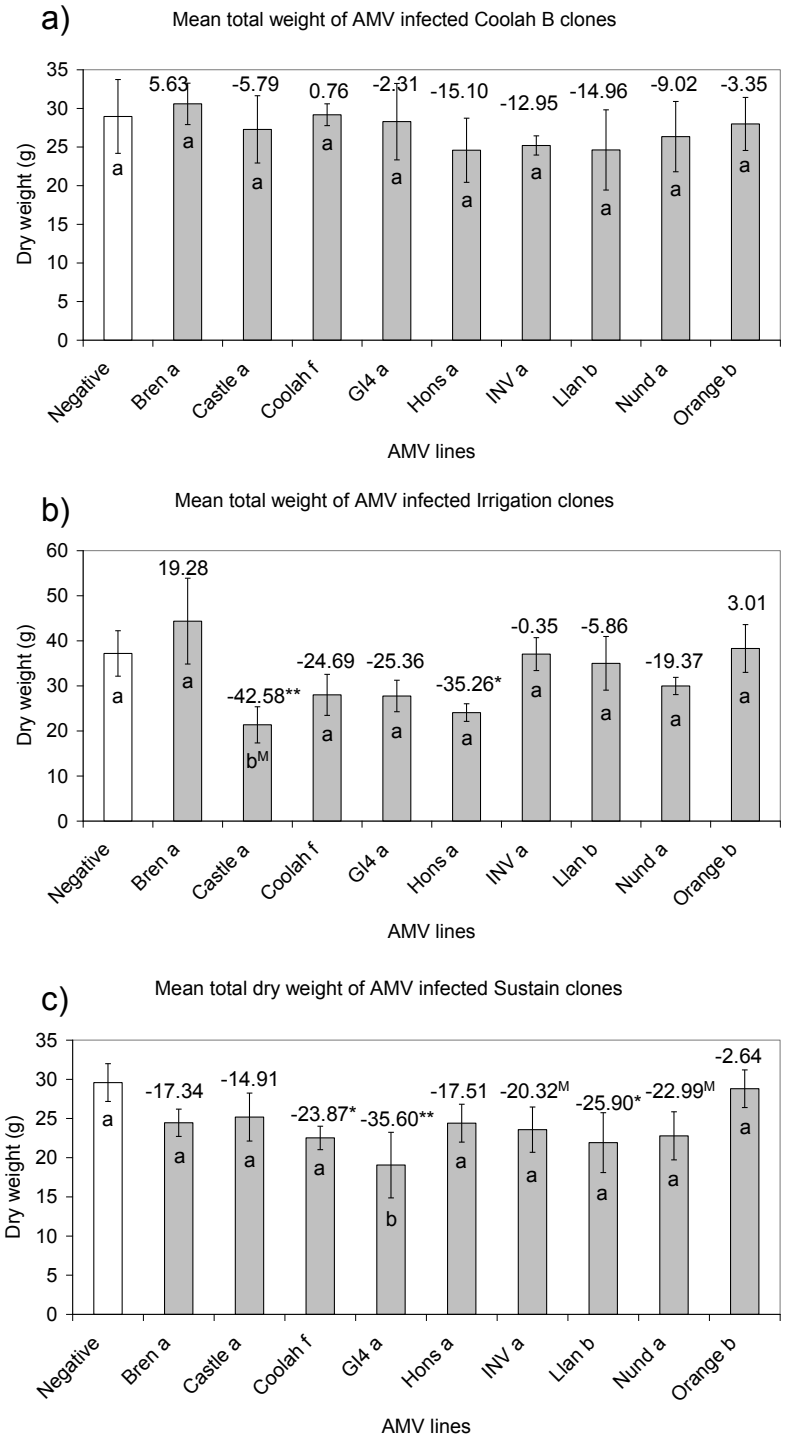


Figure 4.10: Two commercial (*Irrigation* and *Sustain*) and one naturalised white clover line (*Coolah B*) infected with nine AMV lines. Mean total weight of AMV infected of AMV infected *Coolah B* clones a), *Irrigation* clones b) and *Sustain* clones c) measured at census 3 (after 3 months of growth) is presented. White bars represent the mean value of virus negative (V-) clones and grey bars represent the mean value of clover lines infected with individual AMV lines (V+). The standard error of the mean is represented by error bars. The percentage relative virus effect (RVE= ((V+/V-)-1)*100) for each virus line is reported above each bar. Significant V-/V+ contrasts are indicated by * (*P* = 0.01-0.05), ** (*P* = 0.001-0.01) or *** (*P* <0.001). Contrasts with marginal *P* values (0.05-0.1) are indicated by M. Groups with Dunnett test (95% confidence interval) results significantly different from V- are identified by different letters. Groups with marginal Dunnett test results (90% confidence interval) are identified by a different letter followed by M.

The AgDryWt (Figure 4.9) and Totwt (Figure 4.10) results of the two commercial and one naturalised clover line infected with the nine AMV lines were plotted by individual virus line. The figures provide an indication of the diversity of aggressiveness found within Australian AMV lines. These variables were selected because virus model effect was significant for AgDryWt ($P = 0.02$) and marginal for Totwt ($P = 0.065$) (Table 4.7). Post-hoc Dunnett's test (95% confidence interval) indicated that the growth of most AMV infected groups were not significantly different from virus free plants. Only clover lines *Irrigation* and *Sustain* displayed some differences as a result of AMV infection, with three clover x virus line combinations significantly different to the negative control group:- AgDryWt *Irrigation* x *Coolah f* (RVE = -37.5%); *Sustain* x *GI4 A* (RVE = -36.6); and Totwt *Sustain* x *GI4 A* (RVE = -35.6%). In addition, Dunnett's tests with a 90% confidence interval indicated that the combinations *Irrigation* x *Coolah f* and *Irrigation* x *Hons* had marginally different AgDryWt, and that *Irrigation* x *Castle a* had marginally different Totwt than that of the control group. When results are analysed with simple V-/V+ contrasts, more clover x virus combinations were found to be significantly different from control groups (Figure 4.9 and 4.10).

The clover x virus model effects from analysis of count data, BranchNo, PriStoNo and FHTot, (collected at all census dates) by general linear mixed model analysis (Poisson distribution, logarithm link function) were not significant (Table 4.10). The clover x virus interaction was marginal for BranchNo at C2 ($P = 0.08$) and PriStNo at C3 ($P = 0.05$). Although generally not significant, the RVE for these growth parameters was highly variable, ranging from +7.4% to -34.8%. AMV had a consistently negative impact on PriStoNo (RVE = -25.8 to -11.0% across census dates) and FHTot (RVE = -34.8 to -12.8%). Virus model effect was only significant for PriStNo ($P = 0.004$), however clover line effects were significant for all variables at all census dates (except FHTot at

C3 $P = 0.3$), again indicating the differences in growth of the different clover genotypes.

4.3.2.4 Supplementary study

A supplementary trial was performed to confirm the repeatability of the growth trial by including the AMV line *Bren a* and local clover line *Bren F* (included in the original growth trial) and to test the impact of local AMV line *Stoney a* on the naturalised local clover line *Stoney D,* as this virus-clover combination was unavailable for the original trial (see Growth trial design in the Methods section). Data was analysed using the same methods as were used for the data from naturalised clover lines inoculated with local AMV from the original trial. Results are presented in Appendix 4.3 and 4.4. Briefly, the magnitude of the growth variable means measured for *Bren F* in the supplementary study were often different than the means observed in the original study. However, the RVE of growth variables measured for *Bren F* in the supplementary study were comparable to that observed for *Bren F* in the original growth trial. The RVE for *Stoney D* was often lower than that for *Bren F* and was commonly positive, even when *Bren F* displayed a negative RVE [for example Rootwt and AgDryWt (Appendix 4.4a and b), InLeng, Lfwid, StolL and BranchNo (Appendix 4.4e, i and l)]. Although generally not significant, the RVE for the growth parameters for both clover lines were highly variable, ranging from +200.1% to -100.0% (FHTot) (Appendix 4.4).

Significant clover x virus x time model effect was observed for StolL ($P = 0.011$), with marginal effects for Lfleng ($P = 0.063$), Lvs ($P = 0.099$) and Maxht ($P = 0.065$) (Appendix 4.3b). The clover x virus interaction was important for this study, with significant model effects for StolL ($P < 0.001$), Lfleng ($P = 0.017$), Inleng ($P = 0.024$), FHTot at C2 ($P < 0.001$), and marginal effects for Rootwt ($P = 0.101$), Rsratio ($P = 0.077$), Lfwid ($P = 0.056$), BranchNo at C3 ($P = 0.066$) and PriStNo ($P = 0.064$) (Appendix 4.3c and d). These results indicate that growth of the traits measured were often dependant on the specific virus-clover

combination. This is shown in Appendix 4.4e-m, where means of the individual clover are plotted. In summary, the results from the supplementary trial are consistent with those from the original trial in that: time is an important factor, there was diversity in individual virus-clover combinations, clover model effects were generally significant and AMV generally had a negative impact on clover growth.

4.4 Discussion

The study presented in this chapter is intended to determine the impact of AMV on the growth and morphological characteristics of commercial and naturalised populations of white clover (*Trifolium repens*) collected from a range of habitats in south-eastern (SE) Australia, and uses this information to infer the potential for enemy release of *T. repens* populations should AMV-resistant transgenic *T. repens* cultivars be intentionally released into this region.

4.4.1 Susceptibility of Trifolium repens to Alfalfa mosaic virus infection

Trifolium repens has a history of variation in the level of resistance to disease (Burdon, 1980), with resistance found to foliar fungal diseases such as *Cymadothea trifolii* and *Pseudopeziza trifolii* (Burdon, 1980) and viruses such as *Peanut stunt virus* and ClYVV (Taylor *et al.*, 1995). Some resistance to AMV in *T. repens* has been documented (Johnstone and Chu, 1993, Barnett and Gibson, 1975), however this resistance has not been commercialised. In this study the two commercial and 10 naturalised *T. repens* lines that were challenged with the 10 AMV lines, collected from naturalised *T. repens* from across NSW (Figure 4.1), were highly susceptible to infection by AMV. Indeed, *T. repens* lines exhibit no obvious variability in resistance to infection; all lines were infected in over 90% of inoculation treatments, albeit following numerous inoculations (Table 4.2). Although the role aphid vectors play in this pathosystem should not be overlooked, this data suggests little or no resistance to infection to AMV exists among naturalised and cultivated *T. repens* lines

Sustain and *Irrigation* in Australia, the key reason why transgenic lines expressing virus resistance have been developed.

Although there is low nucleotide diversity of AMV in the geographic region studied (Chapter 3), there is strong evidence of variability in the infectivity of different AMV lines. One line in particular, *Stoney a* (from Stoney Creek in the Southern Tablelands, NSW), infected a lower proportion of plants than the other AMV lines tested (Table 4.2). Other studies provide evidence that AMV is diverse in host range, symptom expression and magnitude (Houston and Oswald, 1953). Similar results have also been observed for ClYVV, where variability in both virus infectivity and host resistance is known to exist (Godfree *et al.*, 2009a).

4.4.2 Impact of Alfalfa mosaic virus on Trifolium repens

Various studies describe the newly unfolded leaves of AMV infected *T. repens* as distorted and chlorotic, and the leaves of older plants as having angular, yellow patches and growing slowly (Kreitlow and Price, 1949, Houston and Oswald, 1953). In contrast to these results, the majority of the AMV-infected plants in my study appeared visibly healthy (Figure 4.5), to the extent where, if pot labels were ignored, it was difficult to visually discern which plants were AMV-infected. Furthermore, no infected plants died during the growth trial. This result was also found in another study of AMV-infected *T. repens* conducted by Gibson *et al.* (1981).

Despite no mortality and the healthy appearance of most AMV-infected plants, the results of this growth trial did demonstrate that AMV has the potential to significantly impact *T. repens* growth. In the majority of cases, AMV reduced most morphological and growth variables of local (from the same location) and non-local (from a separate location) naturalised and cultivated *T. repens* lines. However, the impacts varied greatly and, in a few cases, AMV even positively influenced some growth characteristics. Overall, there were significant mean reductions of up to 35% for some growth variables.

In brief, AMV was found to impact mean total dry weight (TotWt) by -13.8% (naturalised clover) to -18.7% (two commercial and one naturalised clover line), and up to -51.6% for individual clover x virus combinations. Significant impacts on mean above ground dry weight by (AgDryWt) ranged from -13.57% (naturalised) to -16.37% (for commercial and one naturalised clover line), and up to -43.4% for individual clover x virus combinations. In relations to mean leaf number (Lvs), significant impacts ranged from -12.7% to -19.1%, with up to -40.5% for individual clover x virus combinations.

My results were generally consistent with results from previous glasshouse and field growth trials, which also found variable impacts of AMV on *T. repens* growth. Some studies found AMV had a severe impact on growth (Gibson *et al.*, 1981), while others did not find significant yield reductions (Pratt, 1968). Houston and Oswald (1953) found the impact was variable depending on the AMV isolate. AMV is known to reduce growth characteristics and even result in death of other plant species (Latham *et al.*, 2004). Latham *et al.* (2004) found AMV reduced faba bean shoot dry weight and seed yield by 41% respectively, lentil by 74-76% (shoot) and 81-87% (seed), and chickpea by 50% (shoot), 98% (seed), and infection killed some chickpea plants. Where the impact of AMV on the growth variables of *T. repens* was measured in previous studies, no yield reduction exceeded 33% (Gibson *et al.*, 1982, Gibson *et al.*, 1981, Miller, 1962, Houston and Oswald, 1953).

Broadly, in this growth trial the impacts of AMV infection were not as large as those observed by Godfree *et al.* (2009a) for ClYVV. However, there was evidence of host-isolate combination specificity in the growth response of infected plants. The impact of AMV infection on the growth characteristics of clover (naturalised and commercial lines) varied greatly depending on the clover x virus combination. For example, AMV infection resulted in a RVE that ranged from 6% to -43.4% for mean above ground dry weight of naturalised clover infected with local AMV (Figure 4.6a).

When AMV and clover lines are examined individually, naturalised and commercial clover lines show low resistance to infection by AMV when the impacts of nine individual SE Australian AMV isolates infecting three individual clover lines are compared (Figures 4.10 and 4.11). There are obvious differences in the growth response of a clover genotype to the different AMV isolates used in this study, notwithstanding that the growth of clover when infected by the individual virus isolates was not significantly different from virus free clover in many cases.

Although the growth parameters of *T. repens* were generally negatively impacted by AMV, some variables, for example inflorescence number, were inconsistently impacted (Table 4.6), and stolon thickness was generally positively influenced by AMV infection (Table 4.4 and 4.8). No other cases have yet been reported where AMV has had a positive impact on *T. repens* growth. Gibson *et al.* (1982) found the number of stolons was similar in virus infected and virus free plants. AMV infected *T. repens* plants have been described as being so severely affected that they are stunted (shortened stolon internodes and leaf petioles) (Kreitlow and Price, 1949, Houston and Oswald, 1953). In my growth trial many virus-infected plants recorded significantly reduced internode length compared to their virus-free clones (Table 4.4, 2.8 and Appendix 4.1e and 4.4e), indicating that those clover plants were being stunted by AMV infection. Increased stolon thickness is likely to be associated with stunted growth.

4.4.3 Cultivated Trifolium repens

From a primary producer's perspective two characteristics are desired when selecting a *T. repens* cultivar: persistence and productivity (yield), with the latter more commonly described. Campbell (1986) defined "yield" as the measurable crop produce, which can be described by parameters of quality and quantity; and "crop loss" as a reduction in either the quality or quantity of the yield. Productivity traits include above ground dry weight and leaf

number. Persistence traits are more difficult to define. The persistence traits of *T. repens* desired by plant breeders vary, often including leaf length and width, ability to survive stress and stolon characteristics such as length, number and internode length (Lee *et al.*, 1993). Studies by Brink *et al.* (1999) and Archer and Robinson (1989) found that the traits primarily responsible for persistence are seedling regeneration and propagation by stolons, with the latter being more important for clover in SE Australia, except during dry years when seedling recruitment is essential (Archer and Robinson, 1989).

In the absence of virus, many growth characteristics of the different clover lines studied in the trial varied significantly. These results are consistent with previous studies of *T. repens* (Lee *et al.*, 1993, Lane *et al.*, 2000, Miller, 1962). Productivity traits of commercial and naturalised clover lines were generally similar, although in some cases naturalised clover outperformed commercial varieties. The virus-free commercial lines *Sustain* and *Irrigation* generally had lower stolon length and branching, but a higher inflorescence number than naturalised clover. These results suggest that cultivated clover genotypes may have lower persistence than naturalised clover genotypes, except during dry years when seed production may prove more important for survival. Indeed, ecotype/wild-type clover genotypes have been demonstrated to be more persistent than commercial clover cultivars (Bouton *et al.*, 2005), displaying longer stolons with more branching (Brink *et al.*, 1999), traits which are likely to improve vegetative persistence during stress.

4.4.4 Potential agricultural productivity of transgenic Trifolium repens

Estimating the economic losses on farm due to specific legume viruses is difficult because of the influence of other factors such as co-infection with additional pathogens such as viruses and root-rot, environmental influences and competition with other plants (Taylor and Ghabrial, 1986, Barnett and Diachun, 1986, Campbell, 1986). There is no one best method to assess yield impacts resulting from viral infection (Campbell, 1986). I conducted a

glasshouse-based growth trial as these types of experiments (under controlled conditions) can provide strong insights into what will occur in natural conditions (Barnett and Diachun, 1986).

In the majority of cases in the growth trial, AMV had a negative impact on the productivity and persistence traits of the commercial cultivars *Irrigation* and *Sustain*. The combined impact of the nine virus lines on above ground dry weight of *Irrigation* was -16.3% and -20.4% for *Sustain*, and on total dry weight for *Irrigation* was -14.6% and -20.1% for *Sustain* (Figure 4.7a and c).

For both cultivated clover lines there was evidence of host-isolate specificity, with variation in the magnitude of the yield impacts depending on the specific virus-clover combination. Individual AMV lines resulted in a significant impact on the yield of the commercial cultivars of up to -37.5% for mean above ground dry weight and -42.6 for mean total dry weight (*Irrigation* x *Castle a*) (Figures 4.9 and 4.10). However, a few AMV lines (e.g. *Bren a*) had a positive influence on these growth traits (Figures 4.9 and 4.10), suggesting that in some situations the transgenic may not provide any improvement in yield.

AMV is a common virus of white clover grown in agricultural landscapes (Barnett and Diachun, 1986), with previous surveys indicating that up to 100% of *T. repens* plants can be infected with AMV in Australian pastures (Coutts and Jones, 2002). Although yield losses are unlikely to be linearly related with disease incidence (Taylor and Ghabrial, 1986), my results indicate that if GM *T. repens* is resistant to AMV infection it is likely that in most circumstances there would be an improvement in productivity. Assuming there is no productivity loss as a result of the resistance mechanism, the growth trial demonstrated that under glasshouse conditions AMV resistance may provide an average improvement in production of 16.3% and 20.4% (yield of above ground material for stock consumption) for commercial cultivars *Irrigation* and *Sustain*. As the direct extrapolation of results from glasshouse based growth

trials to field situations cannot be made (Barnett and Diachun, 1986), field trials comparing the growth of transgenic and conventional cultivars under pathogen pressure in a range of agricultural environments are required to estimate the potential on farm yield and/or and persistence gains provided by GM *T. repens.*

4.4.5 *Implications for risk assessment of virus-resistant Trifolium repens in south-eastern Australia*

The experiments reported in this chapter were undertaken for the key purpose of assessing the degree of environmental risk posed by AMV-resistant transgenic *T. repens* to native plant communities in NSW, the ACT and Vic. By combining the distributional data for AMV and *T. repens* (Chapter 2), virulence data from the inoculation trial and the population dynamics of *T. repens*, the risks posed to native habitats can be preliminarily evaluated.

Overall, naturalised *T. repens* lines displayed no resistance to infection by locally-derived AMV lines. Naturalised *T. repens* lines were, on average, no more resistant than commercial cultivars, a finding which suggests that clover exposed to AMV in SE Australia is likely to become infected.

During the inoculation trial, one AMV isolate displayed a significantly lower level of infectivity (*Stoney a* from Stoney Creek) than the other nine isolates (Table 4.2), indicating that there are potential differences in the infectivity of AMV in SE Australia. These results suggest that further work is required to test whether variation in AMV infectivity could have an impact on AMV resistance or the durability of resistance.

Given that AMV can have a significant negative impact on the growth of naturalised clover (up to 35%), it is likely that the population size of naturalised clover in some SE Australian habitats is currently being limited by AMV. However, differences in the growth impacts depending on the host-isolate combination indicate that AMV may be more important in controlling the population size of some clover genotypes than others. A plant from Brennan's

travelling stock reserve (TSR) (Table 4.2), *Bren F,* infected with local AMV, *Bren* a, often had the highest RVE (mean above ground dry weight RVE = -43.4%, root dry weight RVE = -50.9% and total dry weight RVE = -51.6%) (Figure 4.6 and Appendix 4.4). However, some plant communities may experience little impact from AMV. When clover lines *Irrigation*, *Sustain*, and *Coolah B* were infected with *Bren a,* the RVE was lower or even positive (Figure 4.9 and 4.10). Another example is the plant *Hons C,* collected from the site Honeysuckle (Table 4.2), where the impact of local AMV (*Hons* a) on the local clover line was relatively low (mean above ground dry weight RVE = 1.4%, root dry weight RVE = -7.8% and total dry weight RVE= -0.1%) (Figure 4.6), but when other clover lines were infected with *Hons a* the RVE was considerably higher (Figure 4.9 and 4.10).

Although *T. repens* is more likely to persist in SE Australia via stolon elongation rather than seedling production (Archer and Robinson, 1989), during dry years and in areas where clover persists by re-seeding, a reduction in flowering as a result of viral infection could reduce population survival (Barnett and Diachun, 1986). Halisky *et al.* (1960) found that AMV infection reduced the number of flower heads by 41% and also greatly reduced the number of seeds produced per flower head. However, my AMV growth trial demonstrated similar results to those found by Godfree *et al.* (2009a) for ClYVV, in that the impact on flower number was inconsistent in direction.

Most other studies have found that AMV reduces leaf number, number of nodes on primary and secondary stolons, secondary stolon number, primary and secondary stolon length, leaf and stolon dry weight, although some have found that AMV did not result in a reduction in above ground dry weight (Barnett and Diachun, 1986). For some clover-virus combinations in the study, the impacts of AMV infection were considerably higher than those found in previous studies. However, generally the average impacts of local AMV infection were similar or lower than previously reported. Gibson *et al*. (1981)

found AMV significantly reduced primary stolon length by -22.9%, stolon number by -21.0%, leaf number -31.0% and above ground dry weight by -33.3%. Miller (1962) found AMV infected clover yields were reduced by up to 30%, with an average reduction of 17%. Results from my study indicate significant impacts of AMV on SE Australian naturalised *T. repens* were up to:- -43.4% $P = 0.002$ (average of -16.37% $P < 0.001$) for above ground dry weight (Figure 4.6 and Table 4.3); +18.6% $P = 0.023$ (average results were not significant) for the number of branches on the longest stolon; -65.4% (average RVE at C1 = -18.6% $P = 0.006$, C2 = -25.2% $P < 0.001$ and C3 = -16.4% $P < 0.001$) for the number of primary stolons; -22.5% $P = 0.023$ (average RVE at C1 = -6.2% $P = 0.334$, C2 = -11.6% $P = 0.015$, and C3 = -10.1% $P = 0.052$) for stolon length; and -41.2% $P = 0.008$ for total leaf number (average RVE at C1 = -12.7% $P = 0.026$ and C2 = -17.9% $P < 0.001$) (Appendix 4.2).

Vegetative material and stolon density have been strongly associated with *T. repens* competitiveness and survival (Bouton *et al.*, 2005, Jahufer *et al.*, 2002). A reduction of these traits in naturalised *T. repens* caused by AMV infection, as demonstrated during the growth trial, suggests that AMV resistance is likely to facilitate an improvement in clover growth in communities infested with AMV. Predicting the magnitude of the impacts on populations and the possible population expansion requires targeted field trials over an environmental gradient in all of the habitat types identified at risk in Chapter 2. Studying AMV infection across environmental gradients is vital to accurately quantify the fitness impacts of infection, as infected and uninfected hosts are known to respond differently to abiotic gradients (Seabloom *et al.*, 2009a, Godfree *et al.*, 2009b).

4.4.6 General implications for the risk assessment of transgenic plants

4.4.6.1 Virus-specific assessment

AMV was less prevalent in naturalised white clover than other pasture viruses ClYVV and WClMV (Chapter 2). All AMV-infected plants in this study

survived in contrast to a study of ClYVV, where the aggressiveness of some ClYVV isolates resulted in significant mortality of *T. repens* (Godfree *et al.*, 2009a). Overall, the growth impacts of AMV were less severe than those of ClYVV, which reduced all *T. repens* growth variables measured (StolL, PriStNo, Lvs, Lfleng, and AgDryWt) by over 20%, with stolon number the most reduced (Godfree *et al.*, 2009a). ClYVV also reduced stolon production by 34 to 67%, and total dry weight and leaflet size by at least 10 to 30% (Godfree *et al.*, 2009a). The differences in the impacts of AMV and ClYVV on *T. repens* confirm a key conclusion from Chapter 2: that the risks posed by pathogen-resistant transgenic plants to non-target communities are virus-specific and detailed risk assessments of individual virus-host pathosystems are essential.

4.4.6.2 Co-infection

The co-infection of *T. repens* with AMV and other viruses is very common in natural (Chapter 2) and agricultural environments (McKirdy and Jones, 1997, Coutts and Jones, 2002) in SE Australia. Miller (1962) demonstrated that infection with both AMV and *Bean yellow mosaic virus* (BYMV) (possibly BYMV = ClYVV) generally reduced clover yields by 33 to 52% (on average 43%), or up to 50% (Houston and Oswald, 1953), which equates to the sum or more of the yield reductions of the two viruses when measured individually. However, some co-infected clover lines had yield reductions similar to those when clover was infected by BYMV alone (Miller, 1962). This masking of AMV symptoms by BYMV indicates that in some cases, where plants are infected with other viruses, resistance to AMV may provide no competitive advantage. Consequently, prior to the environmental release of transgenic pathogen-resistant plants it is crucial that the impacts of the targeted pathogen are studied alone and in combination with other common pathogens of the host.

4.4.6.3 Tiered risk assessment

The results gained from a tiered risk assessment alone for transgenic AMV-resistant *T. repens* would have been limited without the process of habitat

identification and the large-scale field survey undertaken prior to the tiered assessment (Figure 2.1). Without these two additional steps the complexity of the pathosystem would not have been revealed. In addition, simple studies (tier one) would not have shown the variability in impacts of virus demonstrated in this growth trial, and if by chance the virus x clover combinations chosen for tier one tests demonstrated no significant impact on growth [for example, clover genotype *Coolah B* which was rarely significantly impacted by AMV infection (Figure 4.7-10)] then it may have been concluded (erroneously) that GM *T. repens* poses a negligible risk to non-target ecosystems.

The type of exploratory study described in Chapter 2 of this thesis may not prove to be either informative or cost effective for the risk assessment of all transgenic plants. However, in the absence of this preliminary work, cases where there is evidence of naturalisation of the plant species in the intended release area and under the circumstances where the transgenic cultivar may have a competitive advantage (i.e. pathogen resistance) compared to wild-type plants, then the three-tier risk assessment process alone may not provide a sufficiently accurate evaluation of the risks posed to non-target habitats.

4.4.7 Conclusion

The results gained from the work presented in this chapter address tier one of the ecological risk assessment of GM AMV-resistant *T. repens* and indicate that the transgenic is likely to pose a risk to non-target plant communities in SE Australia if released. The growth trial results also indicate that the degree of risk to non-target habitats is likely to differ depending on the virus genotype, host genotype and the specific virus x host combinations present in non-target habitats. This outcome indicates that tier two, and possibly tier three studies, would be required to complete the ecological risk assessment of transgenic AMV-resistant *T. repens.*

5 Synthesis

5.1 Part A: Thesis aims, results and risk assessment process

5.1.1 Thesis aims

Despite decades of observation of the movement of plants to new environments, our knowledge of the plant traits responsible for weediness is still limited (Hulme, 2009, Browne *et al.*, 2007). Therefore, evaluating the potential risks that disease-resistant plants may pose to native habitats is challenging (Dale *et al.*, 2002). Furthermore, the role that plant diseases play in limiting the spatial distribution and abundance of host populations is often unknown, apart from a small number of well-documented cases involving catastrophic diseases such as *Phytophthora cinnamomi* in Australia (Shearer *et al.*, 2008) and *Cryophonectria parasitica* in the USA (Paillet, 2002). In general, the impacts of disease on host plant populations are likely to be more subtle than the above mentioned cases, and may be influenced by factors such as habitat type (Godfree *et al.*, 2009b), host density (Ferrandino, 2005), host-pathogen (H-P) co-evolutionary dynamics (Fargette *et al.*, 2006, Jones, 2006), co-infection (Ford, 1967) and the heritability of resistance traits (Conner *et al.*, 2003b).

Genetic modification (GM) has commonly been used to improve the traits of agricultural plants (James, 1998), including pathogen resistance for pasture plant species (Kaniewski and Thomas, 1993). Several studies with research supporting the risk assessment of pasture species have been conducted (Wang *et al.*, 2004, Cunlife *et al.*, 2004, Kang *et al.*, 2009). However, very few ecological risk assessments have been completed for transgenic pasture species (Bagavathiannana and Van Ackerb, 2010, Sandhu *et al.*, 2008, Sandhu *et al.*, 2009). This thesis investigates the potential risks to non-target ecosystems associated with release of transgenic *Alfalfa mosaic virus* (AMV)-resistant white clover (*Trifolium repens* L.) in south-eastern (SE) Australia by:

1) Determining the extent of white clover populations in native plant communities in a 300,000 km² study region encompassing areas of New South Wales, the Australian Capital Territory and Victoria, and the extent of AMV within these populations;

2) Establishing of the circumstances which are likely to lead to the presence of white clover in native plant communities and infestation of white clover by AMV;

3) Determining the genetic diversity, population structure, the likely source of SE Australian AMV, and an assessment of the evolutionary potential of AMV in this region to overcome coat protein (CP)-conferred resistance in GM *T. repens,* by analysis of the RNA 3 CP sequence of AMV from a SE Australian *T. repens* communities and the CP sequence used for transgenic clover; and

4) Evaluating the impact of AMV on the growth and morphological characteristics of naturalised populations of *T. repens* from a range of habitats in SE Australia, to determine the likely impacts on naturalised *T. repens* populations if AMV-resistant GM *T. repens* cultivars are released.

5.1.2 Summary of thesis results

A survey of 213 sites in over 37 habitat types in 2006-2007 indicate that *T. repens* is a significant weed of many high conservation-value native plant communities in SE Australia (present at 59% of sites visited). Indeed, at the majority of sites (70%) in which white clover was recorded it was found to be abundant or very abundant; moderate and low levels of *T. repens* were only observed at 6% and 20% of sites respectively. AMV, *Clover yellow vein virus* (ClYVV) and *White clover mosaic virus* (WClMV) (two other important viruses of white clover) were detected in 15%, 26% and 16% of tested sites, respectively, containing *T. repens* and were found not to be restricted by region or habitat

type. All three viruses were recorded in endangered and threatened native plant communities, but infestation of community type differed across the three virus species.

AMV was more likely to be infecting naturalised *T. repens* in sites that were located close to agricultural land, contained abundant white clover populations, and were characterised by disturbance. AMV was most prevalent in northern inland NSW, likely reflecting the lower drought severity in this region during the collection period (Murphy, 2007), and/or the high productivity of white clover in northern NSW (Hill 1996). No AMV was found in central or eastern Victoria during the survey; however, the possibility that the extended drought in this region reduced white clover populations during the survey period cannot be discounted (Chapter 2).

Analysis of the CP sequence of 83 AMV isolates from naturalised *T. repens* in SE Australia suggests that AMV isolates display little spatial geographic structure by site, community type or region. The majority of the observed variants can be found on small spatial scales (at individual sites). Furthermore, a phylogenetic study revealed that Australian AMV isolates are not associated with a specific host or geographic origin. Results suggest that SE Australian AMV have low to moderate genetic diversity.

Despite none of the AMV isolates sharing an identical CP nucleotide sequence with that used for GM *T. repens*, the majority (71%) of the AMV isolates possessed an identical amino acid sequence to the transgene. The remaining isolates differed by as much as four amino acids from the transgene. The number or nature of amino acid changes required to overcome resistance in GM *T. repens* is uncertain, however any transgenic *T. repens* intentionally introduced in SE Australia is likely to be exposed to all of the AMV variation observed (Chapter 3).

Glasshouse experiments undertaken in this study indicate that SE Australian white clover genotypes are susceptible to infection by AMV, but that variability in the infectivity of AMV isolates is present. The results from my study also demonstrate that AMV has the potential to significantly impact *T. repens* growth. Overall, viral infection reduced most growth parameters, with mean reductions of up to 35% for some variables. There was evidence of host-isolate combination specificity as results varied greatly depending on the individual clover x virus combination. For example, the mean above ground dry weight of naturalised clover lines infected with local AMV lines varied from a relative virus effect (RVE) of 6% to -43.4%. This result indicates that AMV infection may in fact be more important in reducing the host population size of some clover genotypes than for others. The general negative growth response of infected plants suggests that AMV is likely to be a significant pathogen of the majority of white clover populations in SE Australia (Chapter 4).

AMV resistance is likely to confer a fitness advantage for *T. repens* plants (up to 35% for some growth parameters under glasshouse conditions), and although AMV was not found to be an abundant virus (found at 15% of sites with clover) the size of some naturalised clover populations is likely to be limited by AMV as frequency at a site was found to be as high as 95% (31% on average). Therefore, transgenic virus-resistant *T. repens* may pose a risk to native plant communities in SE Australia. Habitats found to be most at risk include:- those located close to agricultural land; those that contain abundant white clover populations; areas characterised by disturbance; or those in the northern NSW region, where AMV was most prevalent (the community types specifically identified at risk are listed in Chapter 2).

These results address stages one, two and three in full and stage four (in part) of a risk assessment of transgenic AMV-resistant white clover for proposed release in SE Australia (see Figure 2.1). My results indicate that *T. repens* is a significant weed of natural environments throughout SE Australia and any

modification of the ecological attributes of *T. repens* has the potential to directly impact on a wide range of ecosystems types in SE Australia. Considerations related to the risk assessment are addressed in more detail in the following section.

5.1.3 The process of ecological risk assessment of Alfalfa mosaic virus-*resistant* Trifolium repens

It is generally agreed that it is difficult to assess the risks and benefits of virus resistant transgenic plants (Thompson and Tepfer, 2010) and, as a result, risk assessment methodology varies. Some authors suggest assessments should consider all "what if" scenarios founded on scientific evidence (Sparrow, 2010), while others state that the aim of a risk assessment is not to totally understand a natural system, but to concentrate only on the data required to make a sound decision (Craig *et al.*, 2008).

This thesis aims to concentrate only on the data that would be needed to make a sound decision, and in accord with the methodology of Craig *et al.* (2008), follows the following risk assessment steps: identification of potential hazards; evaluation of hazards; and determination of the likelihood of the hazards occurring. Craig *et al.* (2008) identifies two types of hazards: Unintended impacts on the target population and unintended impacts on non-target populations. In this thesis the latter is considered within a wider ecosystem context, as recommended by Ghosh and Visser (2008). A schematic diagram illustrating the selected procedure for assessing risks posed by transgenic AMV-resistant *T. repens* to non-target ecosystems in SE Australia is presented in Figure 2.1. The first stage of the risk assessment process involved the identification of habitats likely to contain non-target host populations and of high priority for conservation, for further detailed study. A significant part of this framework involved critical decision-making early in the risk assessment process. Herbarium records, vegetation data and species distribution models (Hill, 1996) were used to identify potential host communities, and these

communities were then prioritised in conjunction with government conservation priorities.

The second stage of the risk assessment involved field surveys, where information on the distribution and abundance of the pathosystem was collected. As the complexity of a pathosystem increases, and/or the geographic distribution of the host species increases, the field survey component of the assessment has the potential to become large. In this study of *T. repens* the pathosystem distribution and abundance data was collected from a diverse range of potentially at-risk habitat types in a 300,000 km^2 area of SE Australia. This approach was then used to inform the development of stage three and the tiered risk assessment (stage four). Stage three and part of stage four were addressed by H-P challenges performed under controlled conditions.

Potential risks specifically associated with GM virus-resistant plants include the impacts of phenotypic changes in H-P interactions, changes in host or virus genotype and the durability of resistance. An evaluation of the potential hazards associated with the deliberate environmental release of GM AMV-resistant *T. repens* are considered in the subsequent sections.

5.2 Part B: Results in a risk assessment context

An outline of how the results from this thesis inform the ecological risk assessment of GM AMV-resistant *T. repens* is considered in the following section. Initially, the potential ecological risks relevant to the transgenic are identified and discussed in detail. Next, the potential for the mitigation of those risks is considered. Then, the management choices for AMV in an agricultural setting are compared, followed by a description of the research required to complete the ecological risk assessment. Finally, the thesis conclusion is presented.

5.2.1 Potential risks due to phenotypic changes in host-pathogen interactions

It could be argued that white clover is not so much of a threat to native plant communities as are other dominant weed species such as serrated tussock (*Nassella trichotoma*) or blackberry (*Rubus fruticosus* aggregate) (Australian Government, 1999). Nevertheless, *T. repens* is a significant weed of a broad range of natural and modified environments in SE Australia, including high quality native plant communities. Therefore, phenotypic changes in H-P interactions such as the naturalisation of transgenic clover genotypes, introgression of the transgene into naturalised clover, or heterologous encapsidation of AMV may result in increased risk to native habitats and agriculture.

My results indicate that *T. repens* can dominate some community types, growing almost as a monoculture, forming mats which may exclude other species (Chapter 2). Godfree *et al.* (2006) found that *T. repens* was one of the most prevalent herbs in *Poa* spp. dominated grasslands and woodlands in SE Australia. It is highly likely that *T. repens* is currently competing with native plant species that have similar patterns of growth, such as herbaceous species which occupy a similar ecological niche: *Cotula alpine, Hydrocotyle sibthorpioides, Pratia pedunculata, Montia fontana, Geranium retrorsum, Ranunculus pimpinellifolius, Veronica gracilis and Haloragis heterophylla* (Godfree *et al.*, 2006).

Trifolium repens is unlikely to directly compete with woody plants in a community. However, nitrogen-fixing invasive species, such as *T. repens*, have been shown to alter nitrogen cycling, resulting in considerable alteration to plant community structure (Ehrenfeld, 2006). It is also likely that the presence of associated root symbionts influence soil microbiota resulting in modified rates of nutrient transformation (Ehrenfeld, 2006). Changes in *T. repens* growth characteristics, such as the naturalisation of new, more productive or persistent elite genotypes with virus resistance have the potential to change native plant community structure.

Generally, my results showed that AMV had a negative impact on plant productivity (for example dry weight and leaf number) and persistence traits (such as stolon length and branching) of white clover. AMV lines reduced the yield of the commercial cultivars, *Irrigation* and *Sustain,* by up to 42.6%. On average, above ground dry weight of *Irrigation* and *Sustain* were reduced by 16.3% and 20.4% respectively. However, in the absence of the virus, the commercial lines were not necessarily better performers than naturalised white clover lines, with naturalised clover outperforming the commercial varieties in some productivity and persistence traits. If clover lines like *Sustain* and *Irrigation* are used for transgenic *T. repens,* the results suggest that in habitats where there is no pathogen pressure, the potential ecological impacts resulting from escape of the transgenic may not exceed the current impacts of naturalised *T. repens*.

Potential phenotypic changes in H-P interactions may also include heterologous encapsidation [encapsidation of viral genome in particles made partially or completely of the CP of another virus (Thompson and Tepfer, 2010)]. This has been demonstrated to occur between two unrelated viruses AMV and *Cucumber mosaic virus* (CMV) (Candelier-Harvey and Hull, 1993). In addition, for cell-to-cell movement, AMV is able to utilise movement proteins from the same family *Bromoviridae* (Sanchez-Navarro *et al.*, 2006). CPs from *Alfamoviruses* and *Ilarviruses* can be interchanged resulting in successful binding and genome activation (Tenllado and Bol, 2000). These studies suggest that heterologous encapsidation is possible between the CP transgene and other *T. repens* infecting viruses.

As the CP is an important determinant of vector type, heterologous encapsidation between the CP transgene and a co-infecting virus could result in a change in vector interactions or specificity (Syller, 2000). For example it may allow aphid transmission of a non-aphid-transmissible virus such as WClMV [mechanical transmission only (Johnstone and Chu, 1993)], which is

commonly found co-infecting *T. repens* with AMV. Viruses with altered transmission properties may pose further risk to current hosts in natural and agricultural systems or could be capable of infecting new hosts.

5.2.2 Potential risks due to changes in host or virus genotype

Potential ecological risks associated with changes in host or virus genotype include transgene introduction into naturalised relatives by outcrossing, and the transfer of genetic material from plant to virus by recombination. This has the potential to result in increased risk to biodiversity and agriculture.

5.2.2.1 Host genotype

Trifolium repens is an obligate cross-pollinator (Thomas, 1987), suggesting that if transgenic clover is intentionally introduced in SE Australia introgression of the transgene conferring resistance to naturalised *T. repens* is highly likely. In addition GM *T. repens* has the potential to become naturalised itself as *T. repens* has a history of escape from agriculture (Holm *et al.*, 1991).

AMV infection of *T. repens* is common in agricultural pastures in Australia (Norton and Johnstone, 1998, McKirdy and Jones, 1997, McKirdy and Jones, 1995, Coutts and Jones, 2002, McLean, 1983), and despite not being as common in naturalised *T. repens* populations, when present, AMV can infect a high proportion (up to 95%) of plants in a population and can have an average negative impact on growth parameters of up to 35%. Resistance to AMV may provide an improvement in naturalised clover growth, as improvements of 16-27% in above ground dry weight, 21-22% in root weight, 13-18% in leaf number, and 6-35% in the length of the longest stolon were demonstrated under glasshouse conditions. These traits are strongly associated with *T. repens* competitiveness and survival (Bouton *et al.*, 2005, Jahufer *et al.*, 2002), suggesting that naturalised clover population size in some habitats is currently being limited by AMV and that resistant genotypes may exhibit increased weediness or invasiveness following relief from pathogen pressure.

In addition, *Trifolium repens* was abundant or very abundant at almost all sites infested with AMV. If we assume the impact of *T. repens* invasion on a native plant community is directly related to plant density, then any niche expansion of naturalised *T. repens* populations is likely to result in a significant negative impact in some habitats types.

Despite the demonstrated impact of AMV on naturalised *T. repens* in the glasshouse, the potential risks associated with release of non-target white clover populations from the effects of AMV may be reduced if other viruses are present in non-target populations. During the survey many naturalised clover plants found infected by AMV were also co-infected with ClYVV and/or WClMV. Previous studies have demonstrated that co-infection of white clover with another virus can reduce the symptoms associated with AMV infection (Miller, 1962, Ford, 1967). The masking of AMV symptoms by another virus may mean that in some non-target habitats in SE Australia, where plants are co-infected, resistance to AMV may provide low or no competitive advantage.

5.2.2.2 Recombination

In addition to the risks associated with plant-to-plant geneflow, the transfer of genetic material from plant to virus by recombination has been demonstrated to occur between the mRNA encoded by a viral transgene to an infecting virus (Greene and Allison, 1994), and recombination has been demonstrated between AMV RNA 3 mutants in transgenic tobacco plants (Van Der Kuyl *et al.*, 1991). Risks associated with the transfer of genetic material from the transgene to a virus by recombination can include changes in host range, vector specificity and virulence (Fuchs, 2008). Risks associated with a change in virus genotype were not explored by this thesis as no novel recombinant viruses have been detected to date in GM plants and viral recombination is generally considered a low environmental risk (Thompson and Tepfer, 2010).

5.2.3 Measures to mitigate the risks associated with changes in phenotype or genotype

A component of the risk assessment includes determining if there is a requirement for risk management and identification of the most suitable methods (Craig *et al.*, 2008). Risk management methods suitable for the hazards GM AMV-resistant *T. repens* poses to SE Australian native plant communities are considered in the following section.

5.2.3.1 Mitigation of the risk of potential phenotypic changes in host-pathogen interactions

A detailed knowledge of H-P spatial distribution in relation to the potential release area is a crucial component of any environmental risk assessment of disease resistant plants. As white clover has a history of naturalisation in Australia it is important to consider the geographic relationship of non-target plant communities and agricultural lands where transgenic clover is likely to be grown. Those communities close to agricultural lands, with an abundant host population (often in regions suitable to grow the host commercially) or disturbed areas (often resulting from agricultural activities) are likely to be most at risk following the release of AMV-resistant *T. repens*. If transgenic AMV-resistant *T. repens* is intentionally introduced in SE Australia, then these high risk habitats are likely to be close to the site/s of release and, therefore, the most difficult to protect. Management techniques such as buffer-zones and the destruction of transgenic material have been previously used for field trials of virus-resistant transgenic white clover (Spangenberg *et al.*, 2001), and may reduce the risk of escape of novel virus-resistant genotypes or transgene introgression onto naturalised clover.

Heterologous encapsidation could occur in co-infected plants in the absence of a transgene and, as such, is considered to be a low environmental risk for GM plants (Fuchs, 2008). AMV CP mutations have been demonstrated to interfere with CP dimer formation, the initiation of infection, plus-strand RNA accumulation, virion formation, cell-to-cell movement and systemic spread of

virus within the plant (van Rossum *et al.*, 1997, Tenllado and Bol, 2000), and these mutations could be utilised to reduce the risk of heterologous encapsidation. Sections of the CP transgene related to vector specificity could be modified so that the protein cannot interact with the vector or is unable to form viral particles (Thompson and Tepfer, 2010, Candelier-Harvey and Hull, 1993). Alternatively, a truncated CP gene has been successfully used for potyvirus *Tobacco etch virus* (TEV), with improved resistance and no risk of transmission of transcapsidated particles (Lindbo and Dougherty, 1992).

5.2.3.2 Mitigation of risks associated with changes in host genotype

The biology of *T. repens* suggests that hybridisation would occur between naturalised and GM *T. repens* in SE Australia (Thomas, 1987). Therefore, native species in the at risk sites identified may be negatively impacted if white clover is provided with a competitive advantage, such as virus resistance. Hybridisation risk between naturalised and GM *T. repens* could be reduced by employing management practices such as buffer-zones and the destruction of GM material. Conditions for the field trial of AMV-resistant transgenic *T. repens* conducted by Spangenberg *et al.* (2001) included a two hectare buffer-zone sown with forage legumes that do not inter-breed with *T. repens* such as lucerne, red and Persian clover. Surrah *et al.* (2008) suggests the use of sterile cultivars to minimise the risk of hybridisation between naturalised and GM potato species in the Peruvian Andes. If such techniques are possible for white clover it could also be a way to mitigate risk.

5.2.3.3 Recombination

Plants in natural conditions are commonly infected by two or more viruses (Ford, 1967). Recombination could occur in co-infected plants in the absence of a transgene, however, recombination has only been detected between a few viruses in nature so far (Moya *et al.*, 1993), and is therefore, considered a low environmental risk (Fuchs, 2008). Nevertheless this risk could be further reduced by using plants with resistance conferred by RNA-silencing which are

unlikely to be susceptible to recombination as transgene expression generally results in no detectable RNA. This is particularly the case when non-coding sequences are used for the transgene (Fuchs, 2008). Conditions where there is high selective pressure increase the likelihood of recombinant virus production (Fuchs, 2008), so selective pressure may by reduced by providing non-transgenic host alternatives in the same agricultural setting.

5.2.4 Durability of resistance

Due to the intentional release of only two types virus resistant transgenic plants, long-term data relating to efficacy and durability of resistance is scarce (Thompson and Tepfer, 2010). In some cases promising experimental resistance has failed in the field (Thompson and Tepfer, 2010). It is unknown how successful the CP transgene will be at providing resistance against SE Australian AMV.

The specific amino acid sequence used for transgenic *T. repens* is likely to be important for resistance. Of the collected AMV isolates, 71% of the sequences analysed were found to be identical to the sequence used for the transgene, most likely rendering those isolates avirulent to GM clover. The remaining isolates had up to four amino acids different from the transgene. It is unknown how different the CP sequence needs to be before CP-mediated resistance breaks down. Research by Taschner *et al*. (1994) showed that a wild-type AMV CP provided resistance for transgenic tobacco against an AMV mutant, with one amino acid change, but a mutant (one amino acid change) CP transgene did not provide resistance against wild-type AMV. For various fungal effector proteins, single amino acid changes can result in a change from avirulence to virulence. However, generally the position of the amino acid substitution in relation to the protein structure determines whether the substitution results in a virulence reaction in the host (Dangl and Jones, 2001, Joosten *et al*., 1994, Schürch *et al*., 2004).

It is also unclear if the type of the amino acid substitution would be important in resistance breakdown for AMV-resistant *T. repens*. However, it has been demonstrated that transgenic tobacco plants expressing a *Tobacco mosaic virus* (TMV) CP mutant, produced a protein unable to aggregate and the transgenic plant displayed low resistance to TMV (Bendahmane *et al.*, 1999). Further, tobacco plants with an AMV CP transgene, containing a frameshift mutation were susceptible to infection (Van Dun *et al.*, 1988). All SE Australian AMV isolates, that had at least one CP amino acid different to the transgene, had at least one change in the secondary structure of the protein. Although the nucleotide sequence diversity of AMV in SE Australia was low, the number of variants in the population and the lack of population structure suggests that, even if GM *T. repens* is intentionally released in a small geographic area, it is still likely to be exposed to the diversity present in the SE Australian AMV population. It is possible that there are already enough variants in the population to overcome resistance, and combined with selection imposed by GM *T. repens*, the virulent AMV may quickly spread to render resistance ineffective.

Currently the genetic diversity of AMV appears to be low to moderate in SE Australia. If genetic diversity equates to evolutionary potential, then the adaptive potential of AMV, in response to AMV resistance, is initially likely to be low to moderate in SE Australia. However, an alteration in host dynamics has the potential to change the pathosystem, increasing selection pressures on the virus, and possibly result in the emergence of novel viruses (Fargette *et al.*, 2006). Within-plant resistance, such as transgenic *T. repens*, resulting in a reduction in virus titre or symptoms, places the virus under selection pressure to evolve an increased multiplication rate and may result in an increase in genetic diversity (Bosch *et al.*, 2006). Therefore, there may be an increase in the genetic diversity (and resultant evolutionary potential) of AMV in SE Australia if transgenic AMV-resistant *T. repens* is environmentally released.

5.2.5 Management choices for Alfalfa mosaic virus

Production losses from plant disease can be limited by control measures, however, for each combination of virus/plant/system there is an economic threshold. Only when the threshold is exceeded do the financial losses justify the use of control measures (Jones, 2004a). Control strategies are ideally robust, have limited extra expense and labour requirements, and involve minimal disruption of normal practices (Jones, 2004a). The economic threshold for transgenic white clover is dependent on plant density, incidence of infection, impact of infection on yield and quality, input and labour costs.

AMV is known to be a common virus in white clover in agricultural landscapes (Barnett and Diachun, 1986). It is thought that the commercial release of GM AMV-resistant *T. repens* in Australia has the potential to ease economic pressures on dairy farmers caused by pasture production losses due to AMV infestation (Garrett, 1991). Previous surveys indicate that Australian *T. repens* agricultural pastures can be infested with AMV up to 100% frequency (Coutts and Jones, 2002), and glasshouse experiments indicate that AMV infection generally has a negative impact on the productivity and persistence traits of commercial cultivars (Chapter 4). Estimates of economic losses are needed to understand the costs and benefits of any virus control strategy (Thompson and Tepfer, 2010). However, despite AMV being common in agricultural systems in Australia (McKirdy and Jones, 1997, Coutts and Jones, 2002, McLean, 1983), information regarding on farm yield losses specifically related to AMV infection is scarce. However, because of their potential importance, some AMV management strategies are briefly discussed in the subsequent section.

5.2.5.1 Conventional management methods

A number of non-transgenic methods could be used to control AMV on farm. Organophosphate (dimethoate) and carbamate (pirimicarb) insecticides can be used against aphids responsible for the spread of AMV to *T. repens*. However, resistance of aphids to insecticides is widespread in Australian agricultural

systems (Edwards *et al.*, 2008). When burr medic (*Medicago polymorpha*) was grown in admixture with annual ryegrass (*Lolium rigidum*), AMV infection was reduced by up to 45% and numerous insecticides significantly decreased AMV incidence by up to 87% (Jones and Ferris, 2000). Although results were dependant on host type and virus strain, Kazinczi (2002) determined that the use of pendimethalin delayed the onset of systemic AMV symptoms in *Chenopodium amaranticolor*. Marco (1993) found that spraying *Capsicum annuum* with 1% mineral oil or 10% whitewash lowered the incidence of viral infection by approximately 40%. Biological control of aphids can also be achieved through the use of indigenous predators and exotic parasitoids (Edwards *et al.*, 2008).

5.2.5.2 Management of *Alfalfa mosaic virus* with transgenic *Trifolium repens*

Resistant plant varieties are generally desired when resistance is not associated with undesirable qualities or characteristics. Ideally a virus resistant cultivar would cost no more than the management of existing cultivars (Taylor and Ghabrial, 1986). The use of control measures that are costly or disrupt normal practices are rarely feasible, except for high value crops (Jones, 2004a). Transgenic *T. repens* may have the potential to control AMV more effectively than conventional management techniques. However, it is likely that there will be higher costs associated with the price of GM seed, adherence to regulatory requirements and changes to established on-farm practices.

Analysis of GM wheat in the USA indicated that commercialisation may result in an overall economic cost (Johnson and Gary Vocke, 2005). Analysis of transgenic cotton in China indicated that, due in part to the high cost of GM seed, *Bacillus thuringiensis* (Bt) cotton is an economically inferior crop protection strategy when compared to other low cost methods (Pemsl and Waibel, 2007). Furthermore Chow *et al.* (2010) found that the costs of producing vitamin A by GM fortification of mustard was almost five times more expensive than supplementation. Conversely, other transgenic species, such as

cotton, soybean, corn and canola have proven extremely successful worldwide (James, 2003). To ensure growing GM clover is worthwhile on-farm, the potential yield and/or and persistence gains must be measured by comparing GM and conventional cultivars in a range of agricultural environments. It would also be advantageous to consider the possible negative trade impacts, and potential increased financial and regulatory costs associated with GM clover.

For the purpose of the experiments undertaken in this thesis, it was assumed that there is no cost associated with CP-mediated resistance and no change in the nutritional value of *T. repens*. Although it has been argued that fitness costs associated with transgenes are rare, numerous studies of transgenics have demonstrated a measurable fitness cost. A fitness cost has been detected in insect resistant transgenic rice under low insect pressure (Xia *et al.*, 2010), in fruit production by cold tolerant transgenic *Arabidopsis thaliana* (Jackson *et al.*, 2004), in fungus resistant transgenic silver-birch (Pasonen *et al.*, 2008), and in leaf-rust resistant transgenic wheat (Romeis *et al.*, 2007). It is unknown if there are fitness costs associated with the CP transgene used for white clover.

5.3 Research required to complete the ecological risk assessment

As previously discussed there are many issues which could be scientifically analysed in the process of assessing the risk of commercial release of a transgenic. This thesis has concentrated only on producing the data required to make a sound decision based on the significant risk factors. The key issue for decision makers is deciding when the data is sufficient to complete the risk assessment. This thesis has identified and evaluated the potential hazards, and evaluated the likelihood of these unintended hazards being realised in non-target populations in SE Australia. White clover is a significant weed of natural habitats in SE Australia and any changes in *T. repens* growth characteristics are likely to directly impact a wide range of ecosystem types. Transgenic virus-resistant *T. repens* could pose some risk to native plant communities in SE

Australia. Resistance to AMV is likely to confer a fitness advantage to *T. repens* plants (up to 35% for some growth parameters under glasshouse conditions), and although AMV was not found as often (found at 15% of sites with clover) infecting naturalised clover as other viruses, ClYVV and WClMV, the size of some naturalised clover populations are currently likely to be limited by AMV as frequency at a site can be up to 95% (31% on average).

To complete a comprehensive risk assessment of the ecological impact of AMV-resistant white clover on non-target plant communities in SE Australia some hazards require further study. Collectively my results indicate that future stages of the risk assessment process should focus on high conservation value habitats with a history of disturbance, large *T. repens* populations, and on sites in close proximity to agricultural land containing hosts for AMV. Additional work required to complete the assessment is briefly outlined in the following sections.

5.3.1.1 Assessing the role of white clover populations in native plant communities and impacts on native species

The thesis results indicate that an important future component of the risk assessment process is an assessment of the role *T. repens* populations play in invaded native habitats and their impacts on native species. As it is unclear whether expanding *T. repens* populations in non-target environments (a likely outcome from the intentional introduction of AMV-resistant clover lines) would cause any significant change in native vegetation, research should be based on field projects, with some supplementary glasshouse competition experiments. Initially, it would be valuable to perform competition trials with native herbaceous species that have been identified to exhibit similar growing patterns to *T. repens* (Godfree *et al.*, 2006), in order to establish the specific impacts *T. repens* could have on these species and potentially the community types they inhabit.

5.3.1.2 Testing *Trifolium repens* seed transmission of *Alfalfa mosaic virus*

Previous study of AMV infected *T. repens* (Latch and Skipp, 1987) and tests undertaken as a component of this thesis suggest that AMV is not seed-borne in *T. repens*. However, seed transmission of AMV has been demonstrated for species that grow in close association with *T. repens* such as *Medicago* spp.(Jones and Pathipanawat, 1989). The possibility of seed transmission in the diverse AMV isolates and *T. repens* genotypes collected in this study has not yet been tested. Seed transmission, even at low levels, would be likely to influence the dispersal and presence of AMV in non-target and agricultural landscapes. Future work could consider investigating seed transmission in more detail during the field based experiments discussed in the following section.

5.3.1.3 Investigating the impact of *Alfalfa mosaic virus* on naturalised *Trifolium repens* populations based on field experiments

The glasshouse growth trial provided valuable information regarding the AMV-clover pathosystem. However, to complete the ecological risk assessment, these experiments should be complemented by field trials investigating the impact of AMV on naturalised *T. repens* populations. Field trials would need to be conducted in conditions with differing habitat quality (over an abiotic gradient) in the habitat types identified most at risk, such as those with both *T. repens* and AMV (see Figure 2.5 for specific habitat types). Similar experiments have been conducted for *ClYVV*, which involved in excess of 1,500 plants and took over three years to assess two environments in the Australian Capital Territory (Godfree *et al.*, 2009b). Results from the survey conducted for this thesis indicate that seven specific medium to very high conservation value habitat types, and numerous low value habitat types, may be at risk following environmental release of transgenic *T. repens*. If the time required to conduct field trials to assess risk for a habitat type following release of GM AMV-resistant *T. repens* is similar to the trials for ClYVV-resistant

T. repens (1.5 years per habitat type), then to assess a minimum of seven habitat types could take 10.5 years if the studies are conducted sequentially.

5.3.1.4 Testing the durability of resistance

There are general concerns that there is a lack of knowledge of the breadth and durability of field resistance of virus-resistant transgenics (Thompson and Tepfer, 2010). To be confident of the efficacy of CP mediated AMV-resistance in *T. repens,* transgenic plants should be subjected in the field to AMV isolates representing the CP diversity present in SE Australia. The nature of AMV interactions with co-infecting viruses, such as ClYVV and WClMV, and the transgene are also likely to be important for long-term resistance stability. An understanding of the nature of these interactions would be a valuable component to a detailed environmental risk assessment.

5.3.1.5 Fitness advantages and costs associated with the expression of virus resistance

Prior to intentional release of GM *T. repens,* field trials should involve testing the extent of any possible fitness costs associated with the transgene, in a range of agricultural environments including under conditions with varying degrees of pathogen load (including co-infection with other common pathogens). Studying viral infection in different environmental conditions is vital to accurately quantify the fitness impacts of infection, as infected and uninfected hosts can respond differently to abiotic gradients (Seabloom *et al.*, 2009a, Godfree *et al.*, 2009b).

5.3.1.6 Assessing the impact of climate change

Results indicate that the range of native habitats in which *T. repens* is naturalised is at least partially limited by climatic conditions or a 'climatic envelope', a finding supported by Hill (1996). Therefore, it is feasible that shifts in climatic zones due to climate change (Thomas *et al.*, 2004), could affect the regions and types of communities where *T. repens* is weedy. Shifts in climatic zones could result in the invasion of *T. repens* into new areas, including

endangered native plant communities. In addition, it is predicted that climate change may increase vector reproduction and survival rates exposing more hosts to viral infection (Yamamura and Kiritani, 1998), and lead to a decline in disease resistance of some hosts (Garrett *et al.*, 2006). It is unknown how climate change would impact the AMV-clover pathosystem. Consequently, a significant shift of climatic zones in SE Australia would necessitate reappraisal of the early stages of the risk assessment process (Table 2.1).

5.4 Conclusion

This thesis demonstrates that *T. repens* is extensively naturalised in native plant communities in SE Australia and, although GM AMV-resistant white clover may reduce yield losses associated with viral infestation on farm, the transgenic poses a potential threat to native habitats including a number of endangered plant communities. The results show that the exploratory study described in this thesis was a crucial component of this risk assessment, and that tiered risk assessment alone may not have accurately demonstrated the risks posed to non-target habitats. The results also illustrate the complexity of conducting ecological risk assessments that involve widespread, invasive pasture species and demonstrates the general need for targeted, habitat- and pathosystem-specific risk assessments. This thesis completes the initial stages of the ecological risk assessment of AMV-resistant *T. repens* and details the work necessary to complete the assessment. A considerable, potentially insurmountable, amount of research is required to complete the ecological risk assessment. I propose that for the risk assessment of pathogen-resistant transgenics such as AMV-resistant *T. repens*, where both the host and pathogen are already established in numerous non-target habitat types and environmental conditions, the research required to complete the risk assessment may well exceed reasonable assessment time-frames and available financial resources. If this is indeed the case for these types of transgenics, then assessors and regulators are left with the question: Can a decision be made

regarding the environmental release of a transgenic, in the absence, not of the knowledge of how to complete the risk assessment, but of an inability to complete the assessment?

6 Appendices

Appendix 2.1: Habitats surveyed

Habitat/location	References
Endangered and threatened plant communities	(Office of Legislative Drafting and Publishing, 2007, Victorian Government, 2007, New South Wales Government, 2008)
Wetlands of National Significance	(Environment Australia, 2001, Hope *et al.*, 2005, Department of Sustainability and Environment, 2006)
Bega Valley	(Keith and Bedward, 1999)
Bogong High Plains	(Wahren *et al.*, 1999)
Subalpine and Alpine wetlands	(Hope, 2002, Whinam *et al.*, 2003, Taranto *et al.*, 2004)
Subalpine and Alpine vegetation	(Gellie, 2005, Walsh and McDougall, 2004, McDougall and Walsh, 2002, Costin, 1957, Wimbush and Costin, 1979)
Barrington Tops National Park	(Zoete, 2000)
Delegate River	(Ladd, 1979, Department of Sustainability and Environment, 2003)
New England Tableland	(Hunter, 2005, Benson and Ashby, 2000)
Mount Canobolas and Orange	(Hunter, 2002)
Temperate Grasslands	(Carter *et al.*, 2003, Environment ACT, 2005)
Gippsland	(Lunt, 1997)
Travelling Stock Reserves	(Rural Lands Protection Board in association with NSW Agriculture., 2001)

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Appendix 2.2: Location of sites with *Alfalfa mosaic virus* (AMV), *White clover mosaic virus* (WClMV) and *Clover yellow vein virus* (ClYVV)

Name	Region	Latitude	Longditude	Community Type[a]	Clover abundance[b]	Virus present[c]
Barrington Track Creek, Barrington Tops National Park	Hunter NSW	S31 57 43.8	E151 26 14.6	NP,9	4	WClMV
Baw Baw (near ski run)	Alpine VIC	S37 50 34.2	E146 16 09.0	NP, 17	3	ClYVV
Box gully	North West Slopes and Plains NSW	S30 21 36.7	E150 02 23.6	7	3	WClMV
Brennans travelling stock reserve	South coast and Illawarra NSW	S36 39 08.7	E149 49 22.2	TSR	3	AMV, WClMV & ClYVV
Carinya revegetation site	North West Slopes and Plains NSW	S30 21 26.9	E150 03 05.0	REV	3	AMV, WClMV & ClYVV
Castletop	North West Slopes and Plains NSW	S30 07 34.4	E150 07 43.9	14	3	AMV, WClMV & ClYVV
Centennial Park	Metropoliton Sydney NSW	S33 53 51.5	E151 13 52.8	3	Only in surrounding lawns	WClMV & ClYVV
Coolah 1	Central Tablelands NSW	S31 45 37.9	E149 42 32.0	15	3	AMV, WClMV & ClYVV
Coolah 2	Central Tablelands NSW	S31 49 16.6	E149 43 30.4	15	3	AMV, WClMV & ClYVV
Cope Hut, Bogong High Plains Falls Creek	Alpine VIC	S36 54 23.1	E147 17 31.1	NP, 10, 16	3 in disturbed areas	WClMV & ClYVV
Cumnock	North West Slopes and Plains NSW	S30 18 42.2	E149 58 15.3	15	3	AMV, WClMV & ClYVV
Currambene creek	Southern Tablelands NSW	S35 54 13.5	E149 35 41.0	9	2	ClYVV
Diggers creek	Alpine NSW	S36 21 37.0	E148 29 15.3	9	4	WClMV & ClYVV
Dry plains	Alpine NSW	S36 05 57.2	E148 57 18.7	9	3	ClYVV
Duck flat 2 travelling stock reserve	Southern Tablelands NSW	S35 08 55.4	E149 34 21.8	TSR	4 in wet or disturbed areas	ClYVV
Emmaville road	North West Slopes and Plains NSW	S29 36 14.6	E151 14 40.0	15	3	ClYVV
Glen Innes 2	Northern Tablelands NSW	S29 38 19.9	E152 04 22.0	15	3	WClMV
Glen Innes 3	Northern Tablelands NSW	S29 38 38.9	E151 59 11.3	8	4	AMV & ClYVV
Honeysuckle	Alpine NSW	S35 09 36.0	E148 27 36.0	15	3	AMV & ClYVV
Inverell roadside	Northern Tablelands NSW	S29 47 02.3	E151 22 42.5	7	3	AMV & ClYVV
Island 2	Alpine NSW	S36 19 24.9	E148 28 30.1	NP, 11	4	ClYVV & WClMV

Appendix 2.2: continued

Name	Region	Latitude	Longditude	Community Type[a]	Clover abundance[b]	Virus present[c]
Jewell's Swamp	Hunter NSW	S33 01 03.6	E151 41 28.5	2	Only in roadside nearby	WClMV & ClYVV
Jibolang travelling stock reserve	South coast and Illawarra NSW	S36 13 26.7	E149 27 11.2	12	1 in low-lying moist drainage areas	ClYVV
Kempsey Dairy Farm Roadside/ Paddock Edge	Mid-North Coast NSW	S30 59 46.2	E152 54 42.9	15	4	ClYVV
Little Llangothlin Montane Lake	Northern Tablelands NSW	S30 04 56.0	E151 46 28.3	WNS, R, 4, 5	4	AMV, WClMV & ClYVV
Mount Canobolas 1	Central Tablelands NSW	S33 20 23.7	E149 01 01.0	NP, 10	3	AMV, WClMV & ClYVV
Mount Canobolas 2	Central Tablelands NSW	S33 20 40.0	E148 58 56.0	NP, 10	1	AMV & ClYVV
Mt Stirling	Alpine VIC	S37 06 22.2	E146 28 15.8	NP, 10	1	WClMV
Native dog travelling stock reserve	Southern Tablelands NSW	S36 40 01.8	E149 16 55.7	13	Only in a dry creek bed	ClYVV
Nundle	North West Slopes and Plains NSW	S31 18 56.8	E151 08 36.8	7	3	AMV, WClMV & ClYVV
Nunnock swamp	Southern Tablelands NSW	S36 42 08.6	E149 26 38.7	WNS, NP, 1	4	ClYVV
Orange roadside	Central Tablelands NSW	S33 18 27.8	E149 02 42.6	15	3	AMV, WClMV & ClYVV
Peppercorn creek	Alpine NSW	S35 35 36	E148 36 63	NP, 9	4 along creek edge	AMV, WClMV & ClYVV
Stoney Creek travelling stock reserve	Southern Tablelands NSW	S35 21 48.4	E149 19 21.3	9	3 in mesic area along the creek	AMV, WClMV & ClYVV
Tallagandra	Southern Tablelands NSW	S35 37 32.3	E149 28 50.2	9	4	AMV & ClYVV
Terry Hie Hie	Northern Tablelands NSW	S29 48 31.5	E151 09 25.6	15	3	AMV, WClMV & ClYVV
Travelling stock reserve Glen Innes 4	Northern Tablelands NSW	S29 38 38.5	E151 57 44.8	6	4	AMV & ClYVV
Walcha travelling stock reserve	Northern Tablelands NSW	S30 49 56.5	E151 32 25.8	6	2	WClMV

[a]Community type: WNS Wetland of National Significance; NP National Park; R Ramsar; RES Reserve; REV Revegetation site; TSR Travelling Stock Reserves; 1 Montane peatlands and Swamps of the New England Tableland, NSW North Coast, Sydney Basin, South East Corner, South Eastern Highlands and Australian Alps (Endangered Ecological Community (NSW)); 2 Freshwater wetlands on coastal floodplains of the NSW North Coast, Sydney Basin and South East Corner (Endangered Ecological Community (NSW)); 3 Sydney Freshwater Wetlands in the Sydney Basin Bioregion (Endangered Ecological Community (NSW)); 4 Upland wetlands of the New England Tablelands and the Monaro Plateau (Endangered (National)); 5 Upland Wetlands of the Drainage Divide of the New England Tableland Bio (Endangered (NSW)); 6 Ribbon Gum, Mountain Gum, Snow Gum Grassy Forest/Woodland of the New England Tableland Bio (Endangered (NSW)); 7 Critically endangered (National) White Box- Yellow Box- Blakely's Red Gum Grassy Woodland and Derived native Grassland; 8 New England peppermint woodland on Basalts and Sediments in the New England Tableland Bio (Endangered Ecological Community (NSW)); 9 Natural Temperate Grassland of the Southern Tablelands of NSW and the Australian Capital Territory (Endangered (National)); 10 Subalpine Woodland; 11 Montane Wet Sclerophyll Forest; 12 Grassy woodlands; 13 Southern Tableland Dry Sclerophyll Forest; 14 Casuarina riparian woodland; 15 Roadside or disturbed communities; 16 Alpine Snowpatch Community (Threatened (VIC)); 17 Disturbed Alpine Areas.

[b]*Trifolium repens* abundance: 0= 0, 1= low level, 2= moderate level, 3= abundant, 4= very abundant.

[c]All sites were tested for *Alfalfa mosaic virus*, only selected sites were tested for *Clover yellow vein virus* and *White clover mosaic virus* (see Table 2.1).

Appendix 2.3: Potential hosts for *Alfalfa mosaic virus* present in NSW (Hull, 1969, National Herbarium of New South Wales, 2009)

Family	Species[a]
Acanthaceae	*Thunbergia alata* (I)
Aizoaceae	*Aptenia cordifolia* (I); *Tetragonia tetragonoides*
Caryophyllaceae	*Dianthus barbatus* (I); *Stellaria media* (I)
Chenopdiaceae	*Beta vulgaris* (I); *Chenopodium album* (I); *C. ambrosioides* (I) and *C. murale* (I)
Compositae	*Ageratum conyzoides* (I); *Calendula officinalis* (I); *Carthamus tinctorius* (I); *Chrysanthemum* spp. (I); *Cichorium endive* (I); *Helianthus annus* (I); *Sonchus oleraceus* (I)
Cruciferae	*Brassica oleracea* (I); *B. rapa* (I)
Cucurbitaceae	*Cucumis melo* (I)
Labiatae	*Ballota nigra* (I); *Nepeta cataria* (I); *Origanum vulgare*; *Stachys arvensis* (I)
Leguminosae	*Crotalaria juncea* (I); *C. spectabilis* (I); *Lespedeza striata* (I); *Lupinus luteus* (I); *Medicago Arabica* (I); *M. lupulina* (I); *M. orbicularis* (I); *M. polymorpha* (I); *M. sativa* (I); *Melilotus alba* (I); *M. indicia* (I); *M. officinalis* (I); *Pisum sativum* (I); *Robinia pseudo-acacia* (I); *Sesbania* spp.; *Trifolium alexandrium* (I); *T. dubium* (I); *T. fragiferum* (I); *T. glomeratum* (I); *T. hirtum* (I); *T. hybridum* (I); *T. incarnatum* (I); *T. pratense* (I); *T. reupinatum* (I); *T. subterraneum* (I); *Vicia faba*; *V. sativa* (I); *V. villosa* (I)
Malvaceae	*Lavatera trimestris* (I); *Malva parviflora*
Moraceae	*Cannabis sativa* (I)
Nyctaginaceae	*Mirabilis jalapa* (I)
Papaveraceae	*Fumaria officinalis* (I); *Papaver somnifera* (I)
Phytolaccaceae	*Phytolacca americana* (I)
Plantaginaceae	*Plantago lanceolata* (I)
Plumbaginaceae	*Limonium sinuatum* (I)
Polygonaceae	*Fagopyrum esculentum* (I); *Rumex crispus* (I)
Portulacaceae	*Portulaca grandiflora* (I); *P. oleracea*
Primulaceae	*Anagallis arvensis* (I)
Resedaceae	*Reseda luteola* (I)
Saxifragaceae	*Mimulus moschatus* (I); *Nemesia strumosa* (I)
Solanaceae	*Capsicum annum* (I); *C. frutenscens* (I); *Cyphomandra betacea* (I); *Datura stramonium* (I); *Hyoscyamus niger* (I); *Lycium ferocissimum* (I); *Nicandra physaloides* (I); *Nicotiana glauca* (I); *N. goodspeedii*; *N. megalosiphon*; *N. occidentalis*; *N. similans*; *N. suaveolens*; *N. tabacum* (I); *N. velutina*; *Petunia hybrida* (I); *Petunia* spp. *(I)*; *Physalis ixocarpa* (I); *P. peruviana* (I); *Solanum capsicastrum* (I); *S. laciniatum*; *S. nigrum* (I); *S. rostratum* (I)
Tropaeolaceae	*Tropaeolum majus* (I)
Umbelliferae	*Ammi majus* (I); *Apium graveolens* (I); *Coriandrum sativum* (I); *Daucus carota* (I)
Verbenaceae	*Verbena officinalis* (I)

[a]I signifies introduced species

Appendix 3.1: Nucleotide sequence alignment of collected Australian *Alfalfa mosaic virus* coat protein (CP) sequences obtained from naturalised *Trifolium repens* and the CP insert (GM_insert) used for genetically modified *T. repens.*

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                          10        20        30        40        50        60
70
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|
Coolah2_13        TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya21_S1      TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya26         TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya39         TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya30         TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Cumnock1_13       TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGCAAAACCGACGAATACTATAC
Cumnock2_6        TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGCAAAACCGACGAATACTATAC
Cast10            TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGNAAAACCGACGAATACTATAC
INR24             TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya18         TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya13         TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
GI4_1             TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_13        TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya10         TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya37         TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya5          TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya24         TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya7_S1       TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Stoney1_10        TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
GI3#2_14          TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Castletop1_3      TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Cumnock1_14       TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Canob1_2          TTACGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Orange12          TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Cast22            TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya28         TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_19        TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
LlanF1_9          TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Terry4            TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Canob2_13         TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
INR11             TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_22        TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Terry6            TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_10        TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_23        TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_3         TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_8         TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_9         TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_5         TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah2_7         TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Carinya7          TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
Coolah1_19        TTGCGCAAAGCTCAACTGCCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATAC
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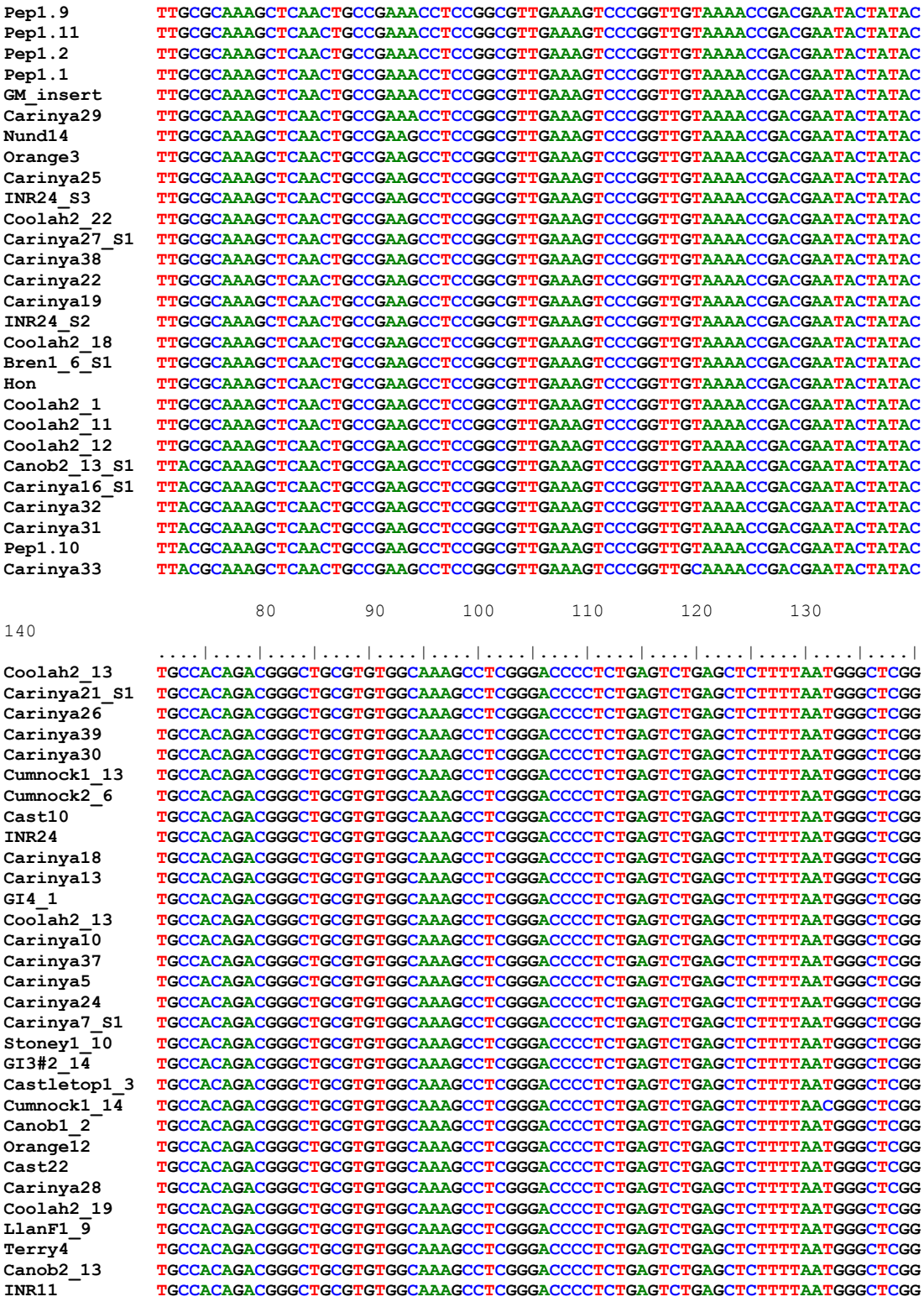
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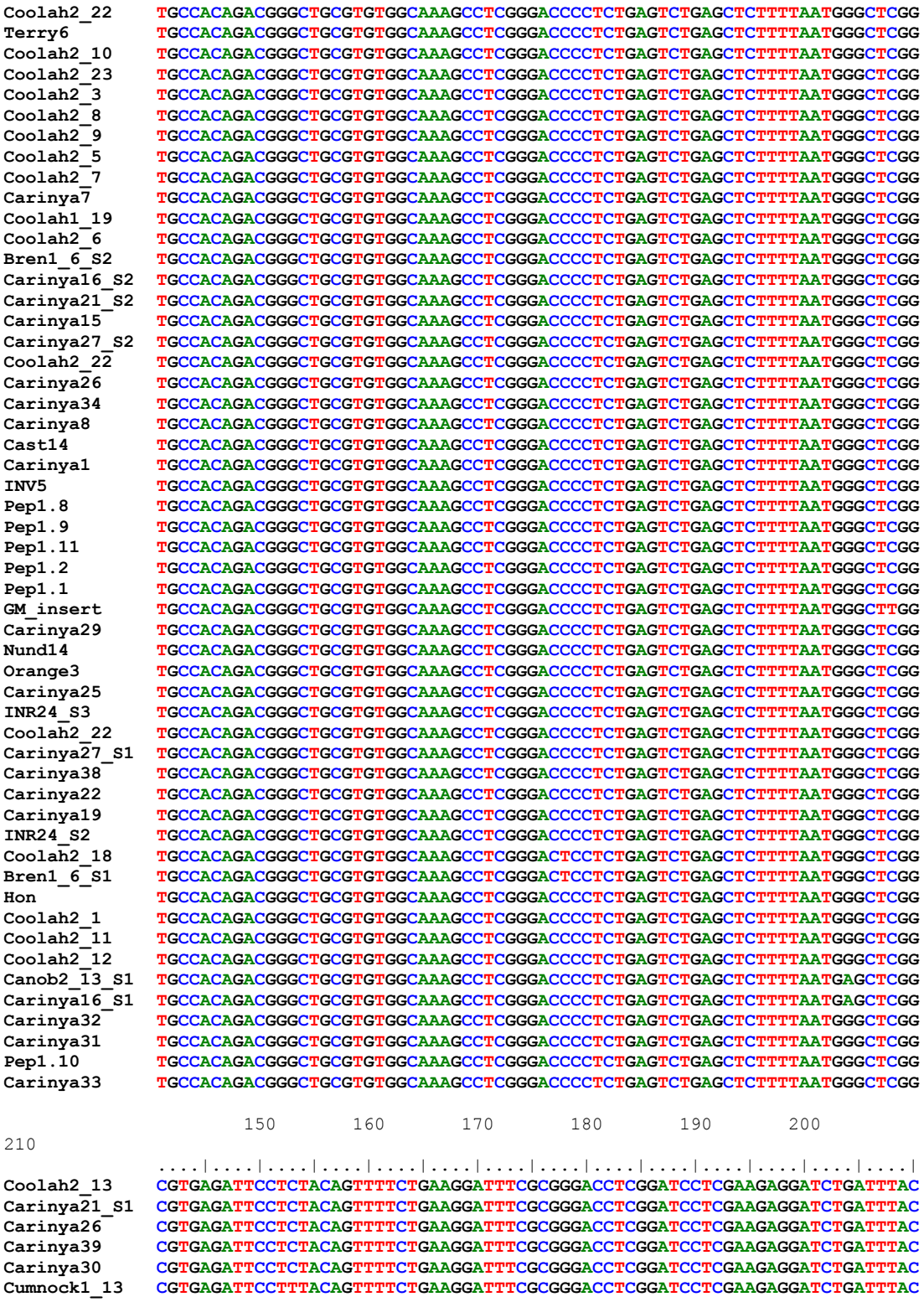
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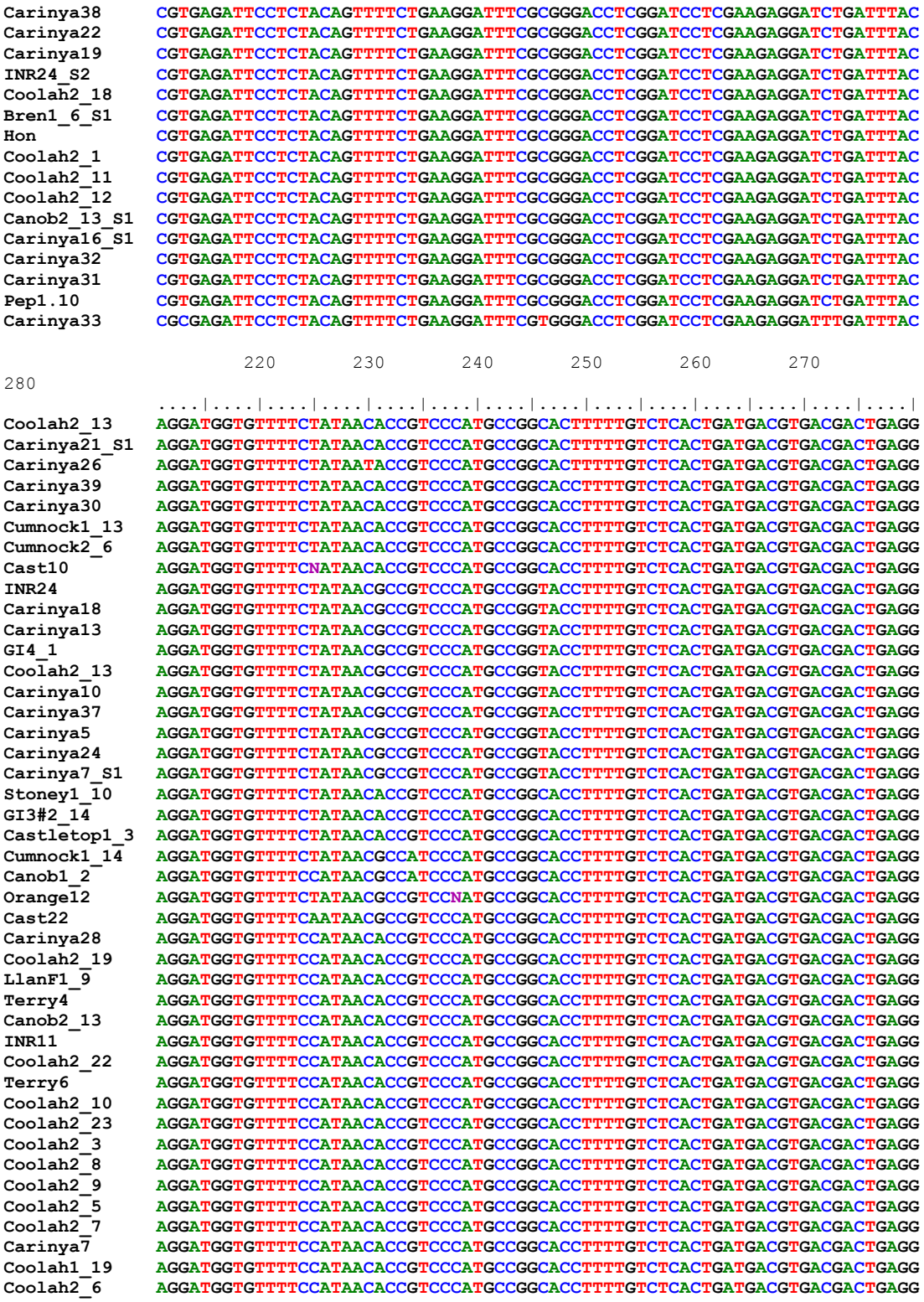
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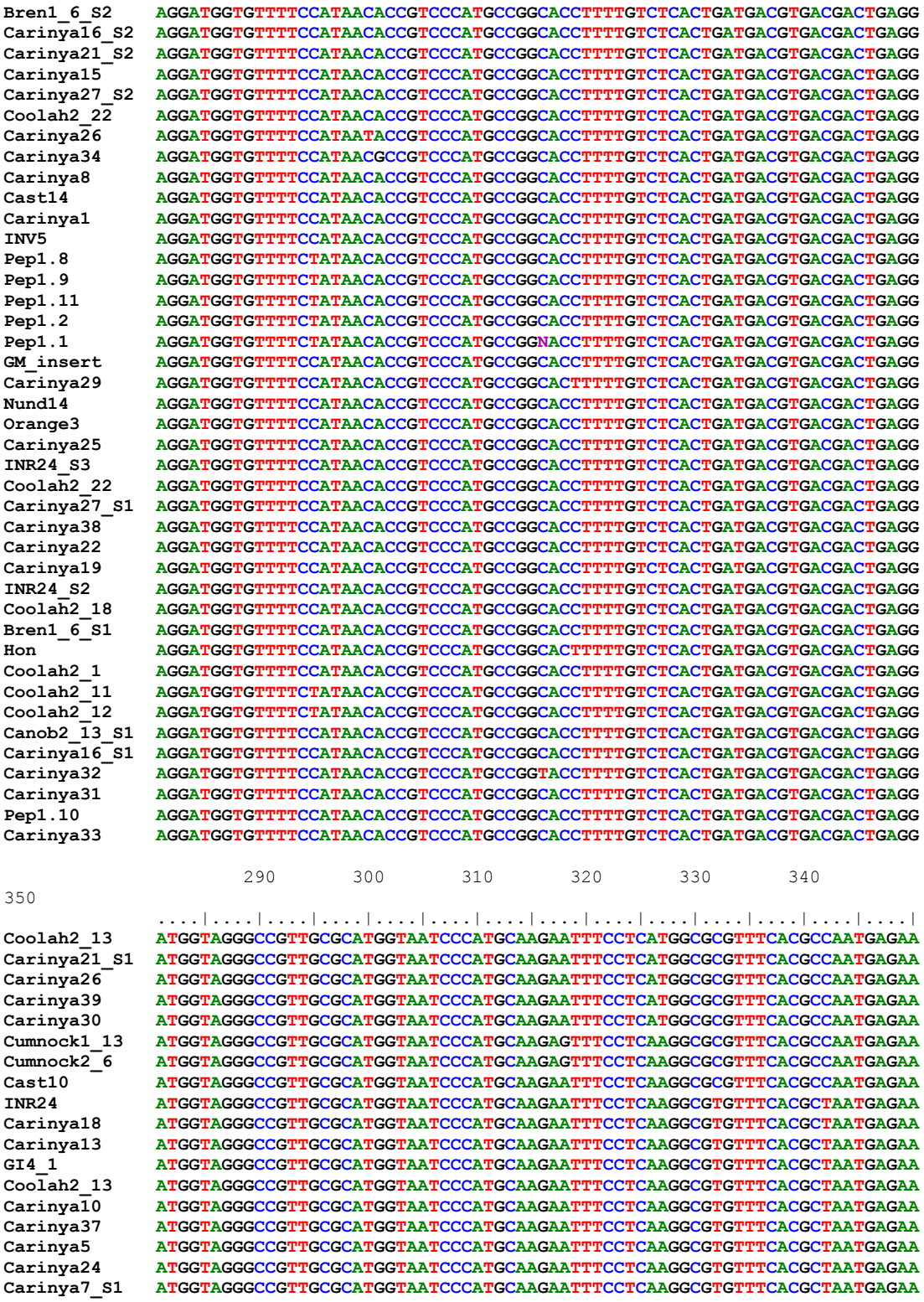
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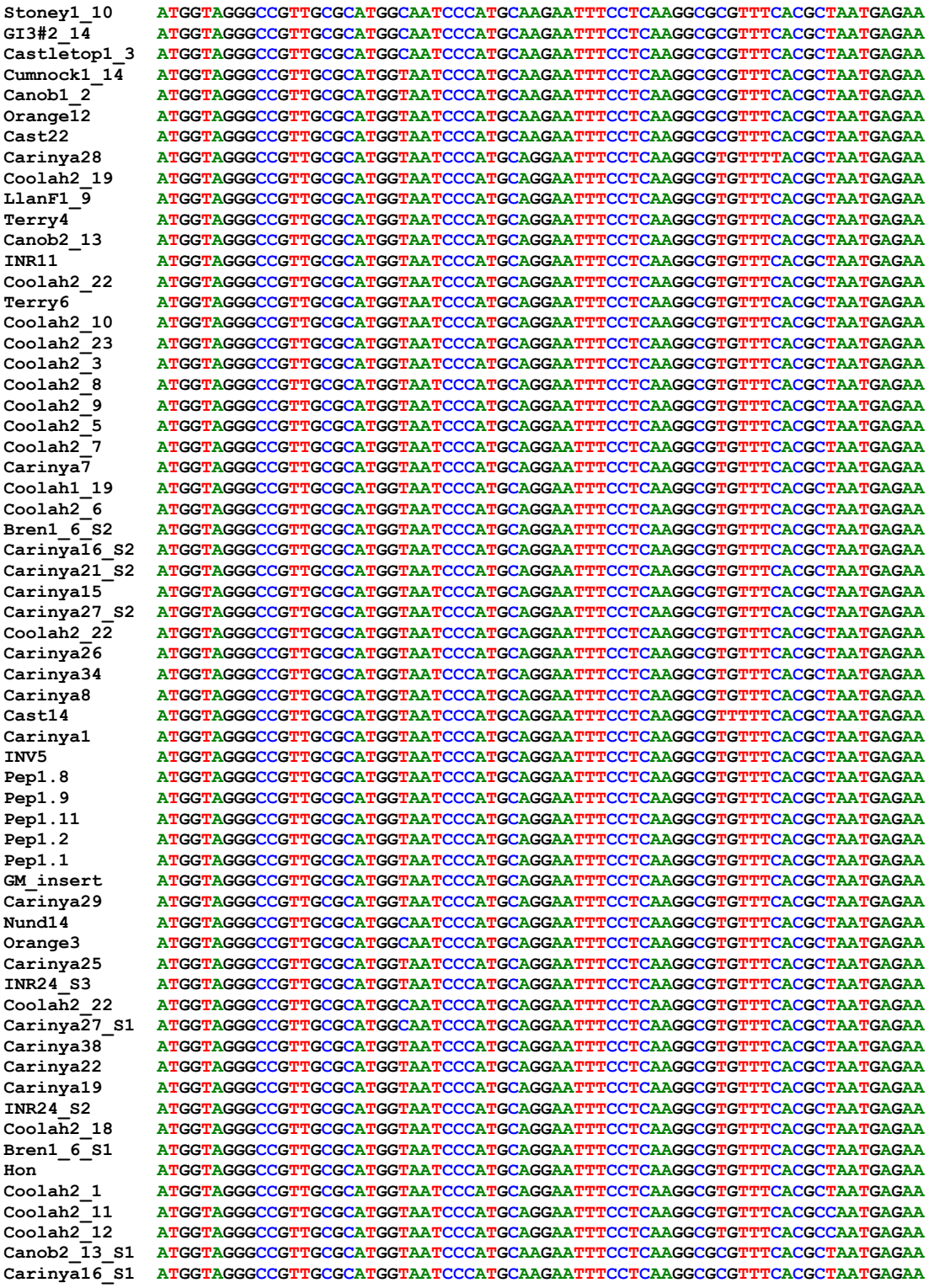
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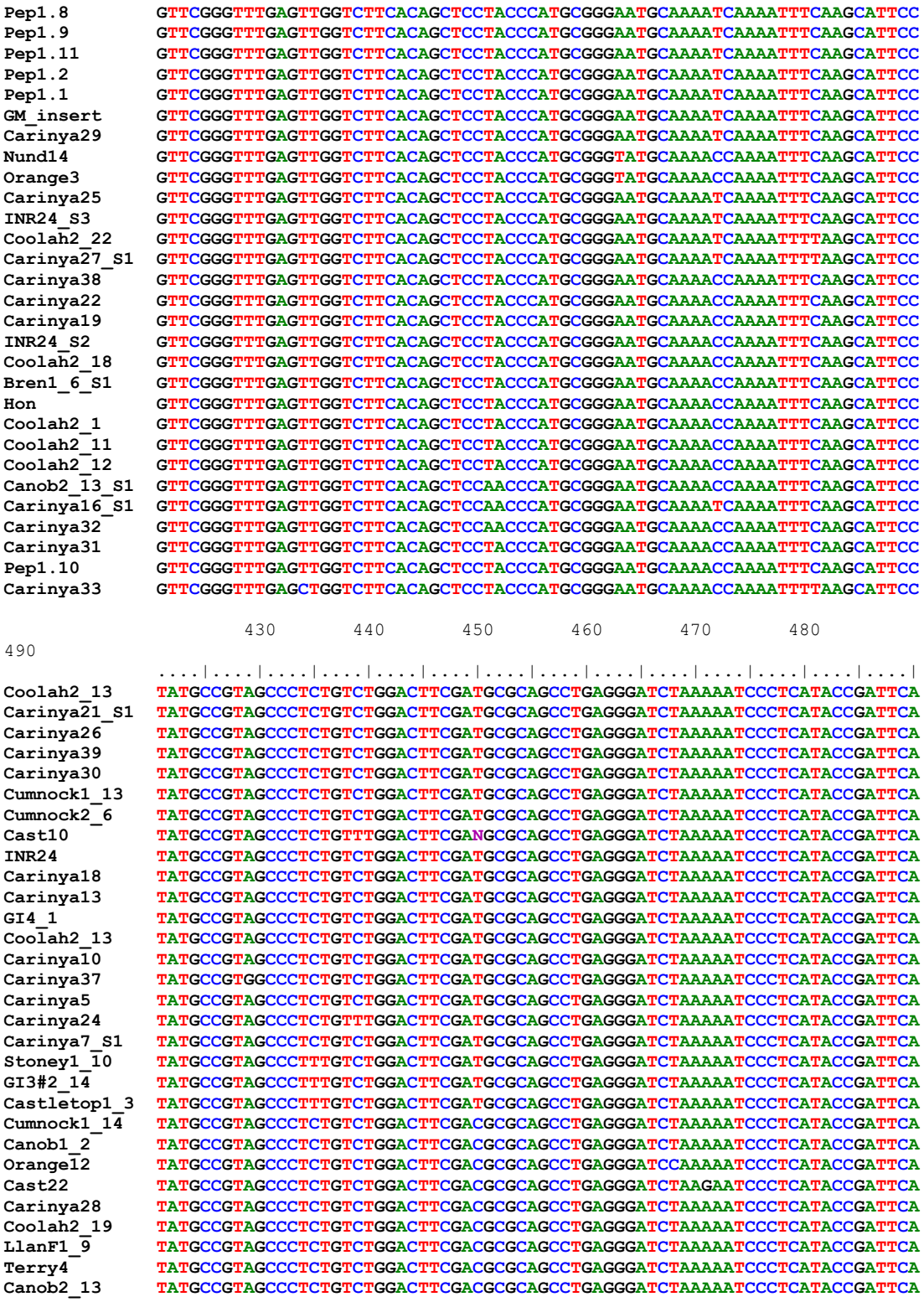
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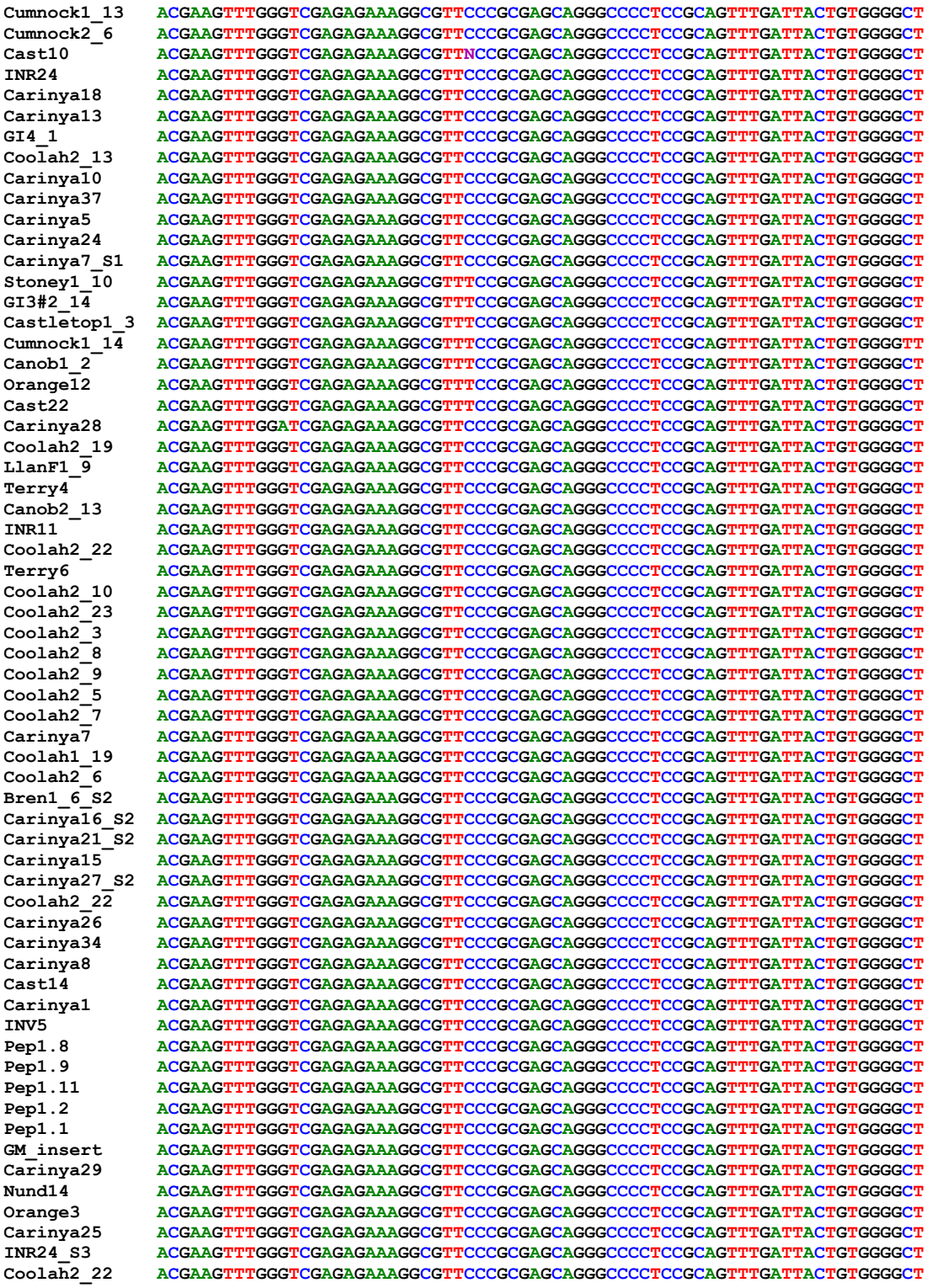
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Bren1_6_S2 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya16_S2 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya21_S2 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya15 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya27_S2 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Coolah2_22 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya26 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya34 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya8 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Cast14 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya1 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
INV5 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Pep1.8 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Pep1.9 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Pep1.11 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Pep1.2 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Pep1.1 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
GM_insert ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya29 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Nund14 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Orange3 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya25 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
INR24_S3 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Coolah2_22 ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT

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Carinya27_S1   ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya38      ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya22      ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya19      ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
INR24_S2       ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Coolah2_18     ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Bren1_6_S1     ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Hon            ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Coolah2_1      ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Coolah2_11     ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Coolah2_12     ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Canob2_13_S1   ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya16_S1   ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya32      ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya31      ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTAATTACTGTGGGGCT
Pep1.10        ACGAAGTTTGGGTCGAGAGAAAGGCGTTTCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT
Carinya33      ACGAAGTTTGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGGCT

                        570
                ....|....|....|....
Coolah2_13     GCTCGACGAAGCTGACGAT
Carinya21_S1   GCTCGACGAAGCTGACGAT
Carinya26      GCTCGACGAAGCTGACGAT
Carinya39      GCTCGACGAAGCTGACGAT
Carinya30      GCTCGACGAAGCTGACGAT
Cumnock1_13    GCTCGACGAAGCTGACGAT
Cumnock2_6     GCTCGACGAAGCTGACGAT
Cast10         GCTCGACGAAGCTGACGAT
INR24          GCTCGACGAAGCTGACGAT
Carinya18      GCTCGACGAAGCTGACGAT
Carinya13      GCTCGACGAAGCTGACGAT
GI4_1          GCTCGACGAAGCTGACGAT
Coolah2_13     GCTCGACGAAGCTGACGAT
Carinya10      GCTCGACGAAGCTGACGAT
Carinya37      GCTCGACGAAGCTGACGAT
Carinya5       GCTCGACGAAGCTGACGAT
Carinya24      GCTCGACGAAGCTGACGAT
Carinya7_S1    GCTCGACGAAGCTGACGAT
Stoney1_10     GCTCGACGAAGCTGACGAT
GI3#2_14       GCTCGACGAAGCTGACGAT
Castletop1_3   GCTCGACGAAGCTGACGAT
Cumnock1_14    GCTCGACGAAGCTGACGAT
Canob1_2       GCTCGACGAAGCTGACGAT
Orange12       GCTCGACGAAGCTGACGAT
Cast22         GCTCGACGAAGCTGACGAT
Carinya28      GCTCGACGAAGCTGACGAT
Coolah2_19     GCTCGACGAAGCTGACGAT
LlanF1_9       GCTCGACGAAGCTGACGAT
Terry4         GCTCGACGAAGCTGACGAT
Canob2_13      GCTCGACGAAGCTGACGAT
INR11          GCTCGACGAAGCTGACGAT
Coolah2_22     GCTCGACGAAGCTGACGAT
Terry6         GCTCGACGAAGCTGACGAT
Coolah2_10     GCTCGACGAAGCTGACGAT
Coolah2_23     GCTCGACGAAGCTGACGAT
Coolah2_3      GCTCGACGAAGCTGACGAT
Coolah2_8      GCTCGACGAAGCTGACGAT
Coolah2_9      GCTCGACGAAGCTGACGAT
Coolah2_5      GCTCGACGAAGCTGACGAT
Coolah2_7      GCTCGACGAAGCTGACGAT
Carinya7       GCTCGACGAAGCTGACGAT
Coolah1_19     GCTCGACGAAGCTGACGAT
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Coolah2_6      GCTCGACGAAGCTGACGAT
Bren1_6_S2     GCTCGACGAAGCTGACGAT
Carinya16_S2   GCTCGACGAAGCTGACGAT
Carinya21_S2   GCTCGACGAAGCTGACGAT
Carinya15      GCTCGACGAAGCTGACGAT
Carinya27_S2   GCTCGACGAAGCTGACGAT
Coolah2_22     GCTCGACGAAGCTGACGAT
Carinya26      GCTCGACGAAGCTGACGAT
Carinya34      GCTCGACGAAGCTGACGAT
Carinya8       GCTCGACGAAGCTGACGAT
Cast14         GCTCGACGAAGCTGACGAT
Carinya1       GCTCGACGAAGCTGACGAT
INV5           GCTCGACGAAGCTGACGAT
Pep1.8         GCTCGACGAAGCTGACGAT
Pep1.9         GCTCGACGAAGCTGACGAT
Pep1.11        GCTCGACGAAGCTGACGAT
Pep1.2         GCTCGACGAAGCTGACGAT
Pep1.1         GCTCGACGAAGCTGACGAT
GM_insert      GCTCGACGAAGCTGACGAT
Carinya29      GCTCGACGAAGCTGACGAT
Nund14         GCTCGACGGAGCTGACGAT
Orange3        GCTCGACGGAGCTGACGAT
Carinya25      GCTCGACGAAGCTGACGAT
INR24_S3       GCTCGACGAAGCTGACGAT
Coolah2_22     GCTCGACGAAGCTGACGAT
Carinya27_S1   GCTCGACGAAGCTGACGAT
Carinya38      GCTCGACGAAGCTGACGAT
Carinya22      GCTCGACGAAGCTGACGAT
Carinya19      GCTCGACGAAGCTGACGAT
INR24_S2       GCTCGACGAAGCTGACGAT
Coolah2_18     GCTCGACGAAGCTGACGAT
Bren1_6_S1     GCTCGACGAAGCTGACGAT
Hon            GCTCGACGAAGCTGACGAT
Coolah2_1      GCTCGACGAAGCTGACGAT
Coolah2_11     GCTCGACGAAGCTGACGAT
Coolah2_12     GCTCGACGAAGCTGACGAT
Canob2_13_S1   GCTCGACGAAGCTGACGAT
Carinya16_S1   GCTCGACGAAGCTGACGAT
Carinya32      GCTCGACGAAGCTGACGAT
Carinya31      GCTCGACGAAGCTGACGAT
Pep1.10        GCTCGACGAAGCTGACGAT
Carinya33      GCTCGACGAAGCTGACGAT
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Appendix 3.2: Origin and host of AMV CP nucleotide sequences used for diversity studies from GenBank (National Center for Biotechnology Information (NCBI)).

NCBI accession no.	Country	Host	Short name used for trees
AF332998	Australia	*Nicotiana clevelandii* (Lab species)	AUS_N.cle
FJ858265	Brazil	Papaya	BRA_Pap
FJ858264	Brazil	Papaya	BRA_Pap2
DQ314750	Canada	Potato	CAN_Pot
DQ314752	Canada	Potato	CAN_Pot1
DQ314749	Canada	Potato	CAN_Pot2
DQ314755	Canada	Potato	CAN_Pot3
DQ314756	Canada	Potato	CAN_Pot4
DQ314754	Canada	Potato	CAN_Pot5
DQ314753	Canada	Potato	CAN_Pot6
DQ314751	Canada	Potato	CAN_Pot7
X00819	England	Lucerne	ENG_Luc
AJ130708	France	Carrot	FRA_Car
AJ130707	France	Pepper	FRA_Pep
AJ130703	France	Tomato	FRA_Tom
K03542	France	Unknown	FRA_Unk
AJ130709	France	Wild Tomato	FRA_W_Tom
AJ130706	Italy	Bean	IT_Bean
AJ130704	Italy	*Portulaca oleracea*	IT_P.ole
Y09110	Italy	Tomato	IT_Tom
AJ130705	Italy	Tomato	IT_Tom2
AB451173	Japan	Potato	JAP_Pot
AF294433	Korea	Potato	KOR_Pot
AF294432	Korea	Potato	KOR_Pot2
AY957607	Mexico	*Leonotis nepetaefolia*	MEX_L.nep
U12509	New Zealand	Lucerne	NZ_Luc
U12510	New Zealand	Lucerne	NZ_Luc2
FJ527748	Serbia	Alfalfa	SER_Alf
EU925642	Serbia	Lilac	SER_Lil
FJ527749	Serbia	Tobacco	SER_Tob
AB126031	South Korea	Unknown	SKOR_Unk
AB126032	South Korea	Unknown	SKOR_Unk2
V00048	The Netherlands	Tobacco	NETH_Tob
AF15716	The Netherlands	Tobacco	NETH_Tob2
AF015717	The Netherlands	Tobacco	NETH_Tob3
AY340070	USA	Alfalfa	USA_Alf
AY340071	USA	Bean	USA_Bean
L00162	USA	Clover	USA_Clo
K02703	USA	Clover	USA_Clo2
M59241	USA	Lucerne	USA_Luc
DQ124429	USA	*Phlox paniculata*	USA_P.pan

Appendix 2.3: Amino acid sequence alignment of collected Australian *Alfalfa mosaic virus* coat protein (CP) sequences obtained from naturalised *Trifolium repens* and the CP insert (GM_insert) used for genetically modified *T. repens.* Identical amino acids are the same colour. One letter amino acid codes are used.

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                        10        20        30        40        50        60
70
                ....|....|....|....|....|....|....|....|....|....|....|....|....|
Coolah2_13      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya21_S1    LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya26       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya39       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya30       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Cumnock1_13     LRKAQLPKPPALKVPVAKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Cumnock2_6      LRKAQLPKPPALKVPVAKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Cast10          LRKAQLPKPPALKVPVXKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
INR24           LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya18       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya13       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
GI4_1           LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_13      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya10       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya37       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya5        LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya24       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya7_S1     LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Stoney1_10      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
GI3#2_14        LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Castletop1_3    LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Cumnock1_14     LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Canob1_2        LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Orange12        LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Cast22          LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya28       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_19      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
LlanF1_9        LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Terry4          LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Canob2_13       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
INR11           LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_22      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Terry6          LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_10      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_23      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_3       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_8       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_9       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
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Carinya7        LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah1_19      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_6       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Bren1_6_S2      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya16_S2    LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya21_S2    LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya15       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya27_S2    LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_22      LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya26       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya34       LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya8        LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Cast14          LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya1        LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
INV5            LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
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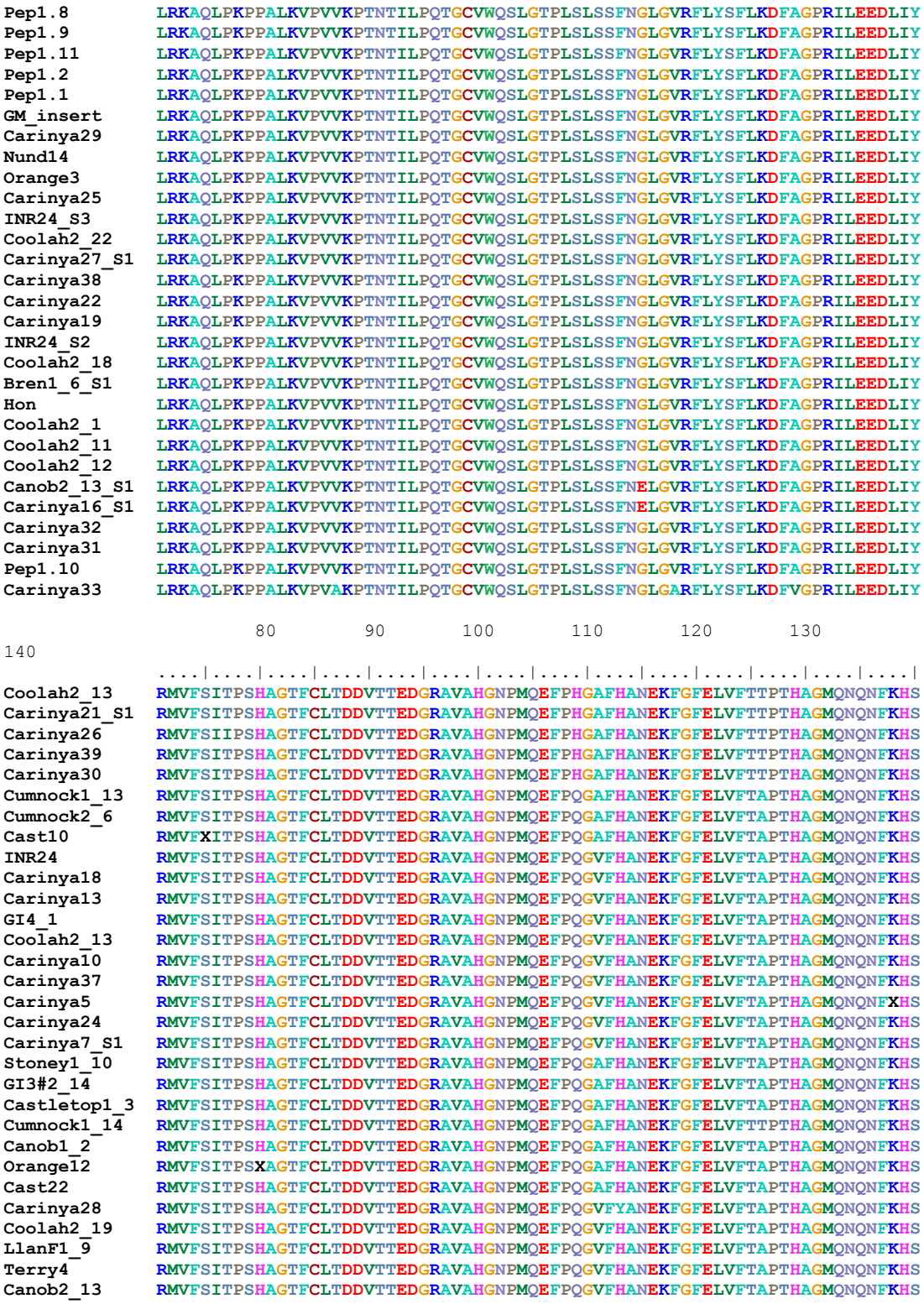
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Carinya29 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Nund14 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Orange3 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya25 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
INR24_S3 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_22 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Carinya27_S1 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
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Coolah2_18 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Bren1_6_S1 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Hon LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_1 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_11 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Coolah2_12 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
Canob2_13_S1 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNELGVRFLYSFLKDFAGPRILEEDLIY
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Carinya32 LRKAQLPKPPALKVPVVKPTNTILPQTGCVWQSLGTPLSLSSFNGLGVRFLYSFLKDFAGPRILEEDLIY
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Carinya21_S1 RMVFSITPSHAGTFCLTDDVTTEDGRAVAHGNPMQEFPHGAFHANEKFGFELVFTTPTHAGMQNQNFKHS
Carinya26 RMVFSIIPSHAGTFCLTDDVTTEDGRAVAHGNPMQEFPHGAFHANEKFGFELVFTTPTHAGMQNQNFKHS
Carinya39 RMVFSITPSHAGTFCLTDDVTTEDGRAVAHGNPMQEFPHGAFHANEKFGFELVFTTPTHAGMQNQNFKHS
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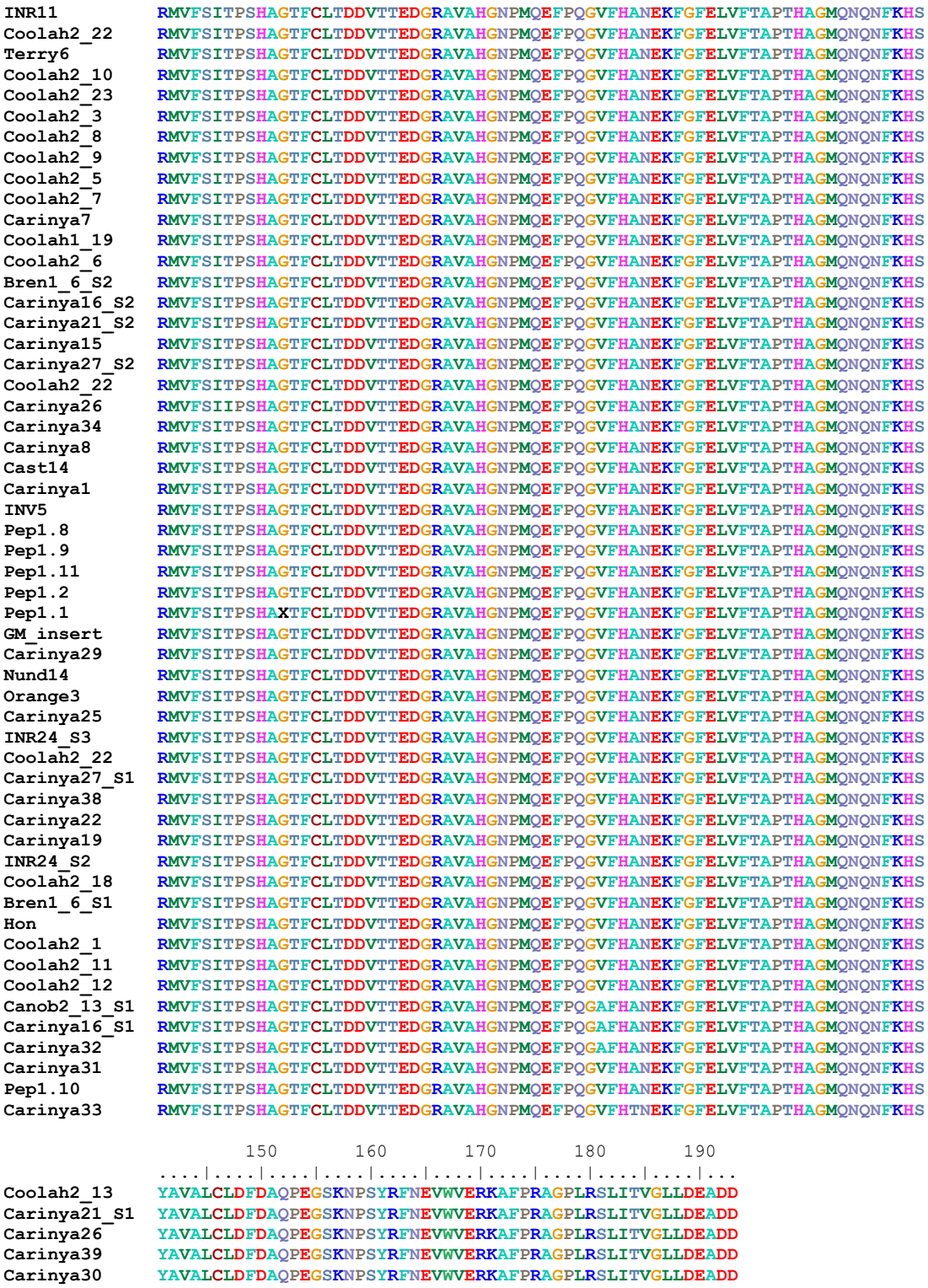
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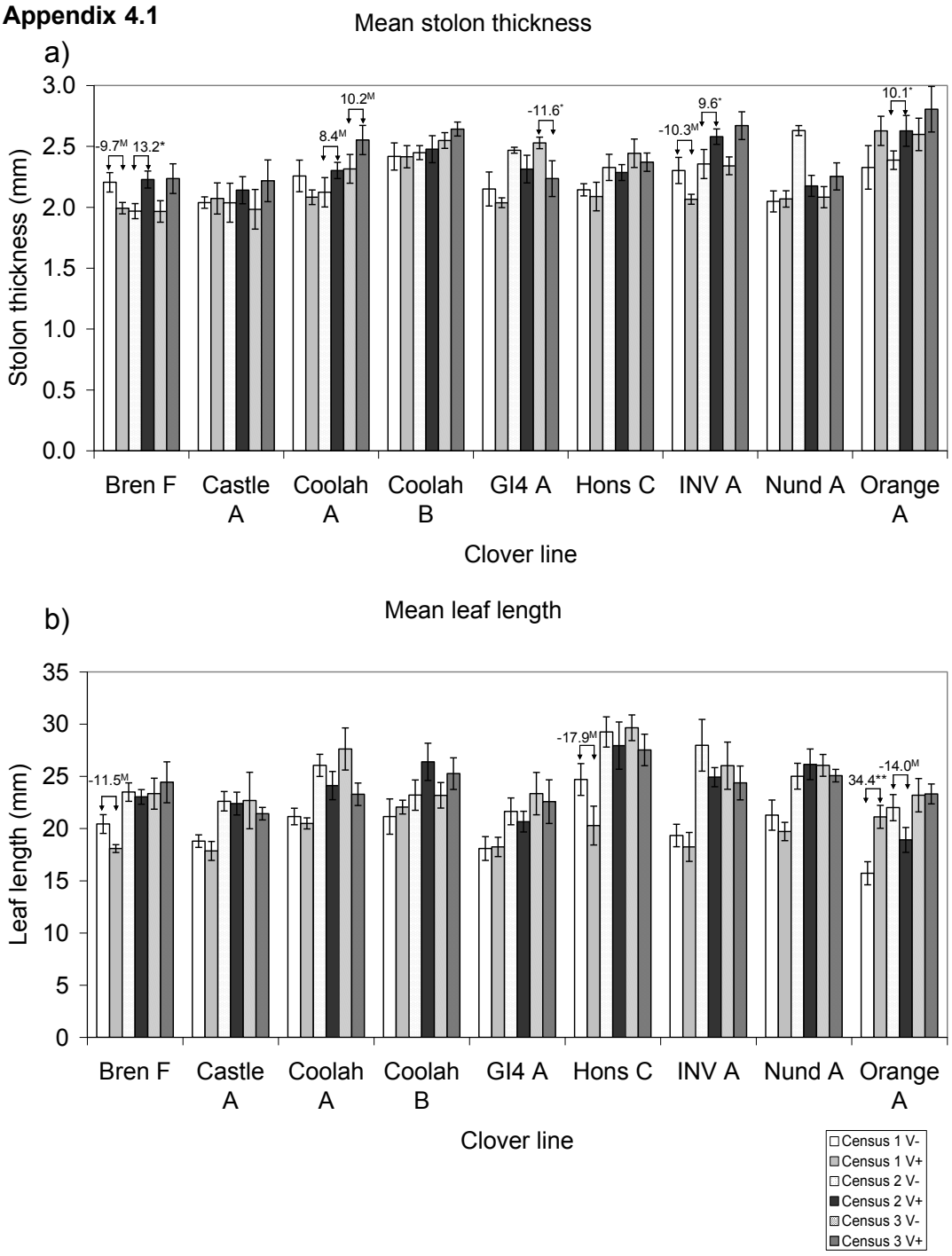
Appendix 4.1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10.2M
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9.6*
10.1*
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Castle A
Coolah A
Coolah B
GI4 A
Hons C
INV A
Nund A
Orange A
Clover line
Mean leaf length
b)
Leaf length (mm)
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30
25
20
15
10
5
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Castle A
Coolah A
Coolah B
GI4 A
Hons C
INV A
Nund A
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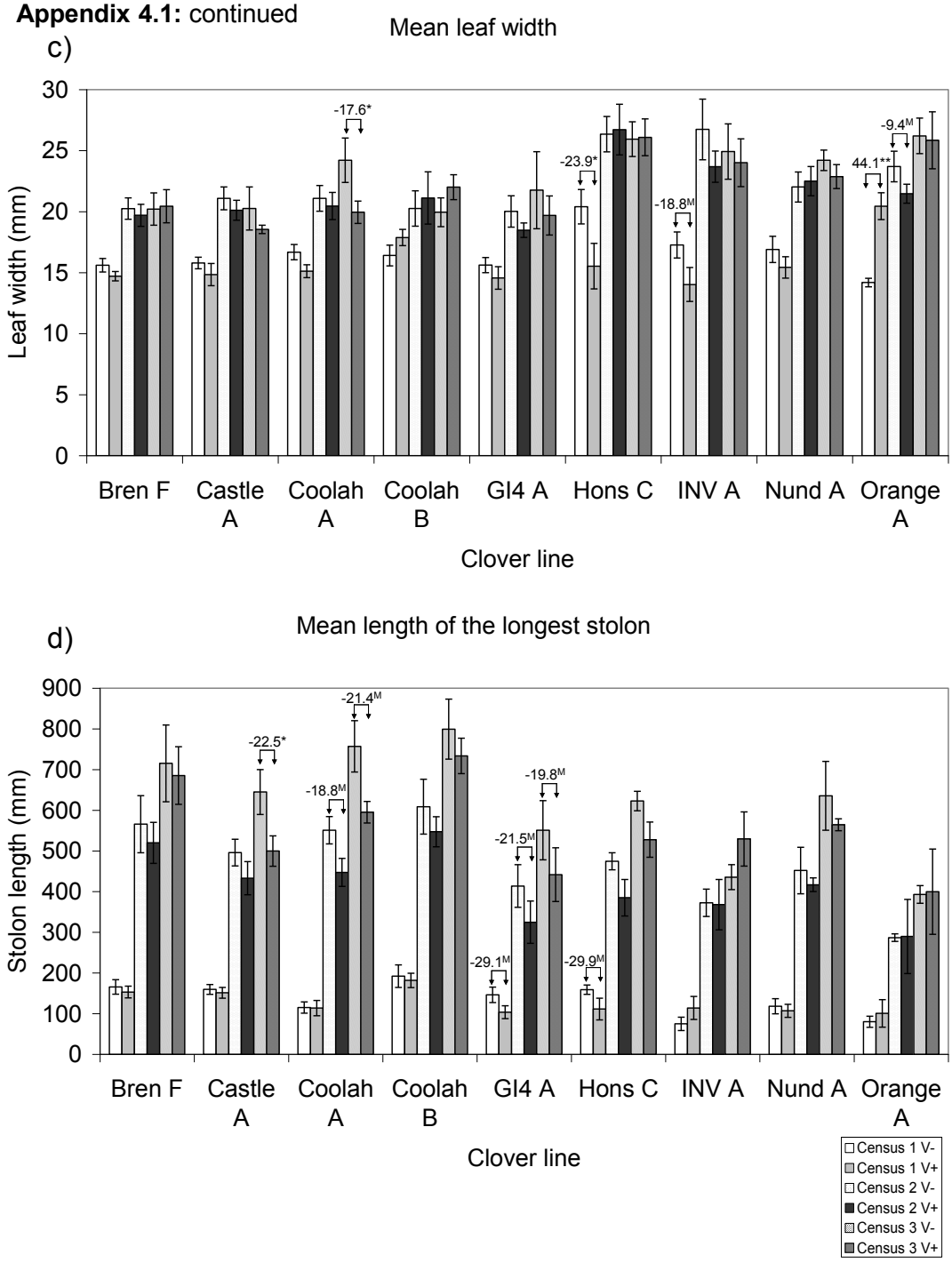
Census 3 V+

Appendix 4.1: continued



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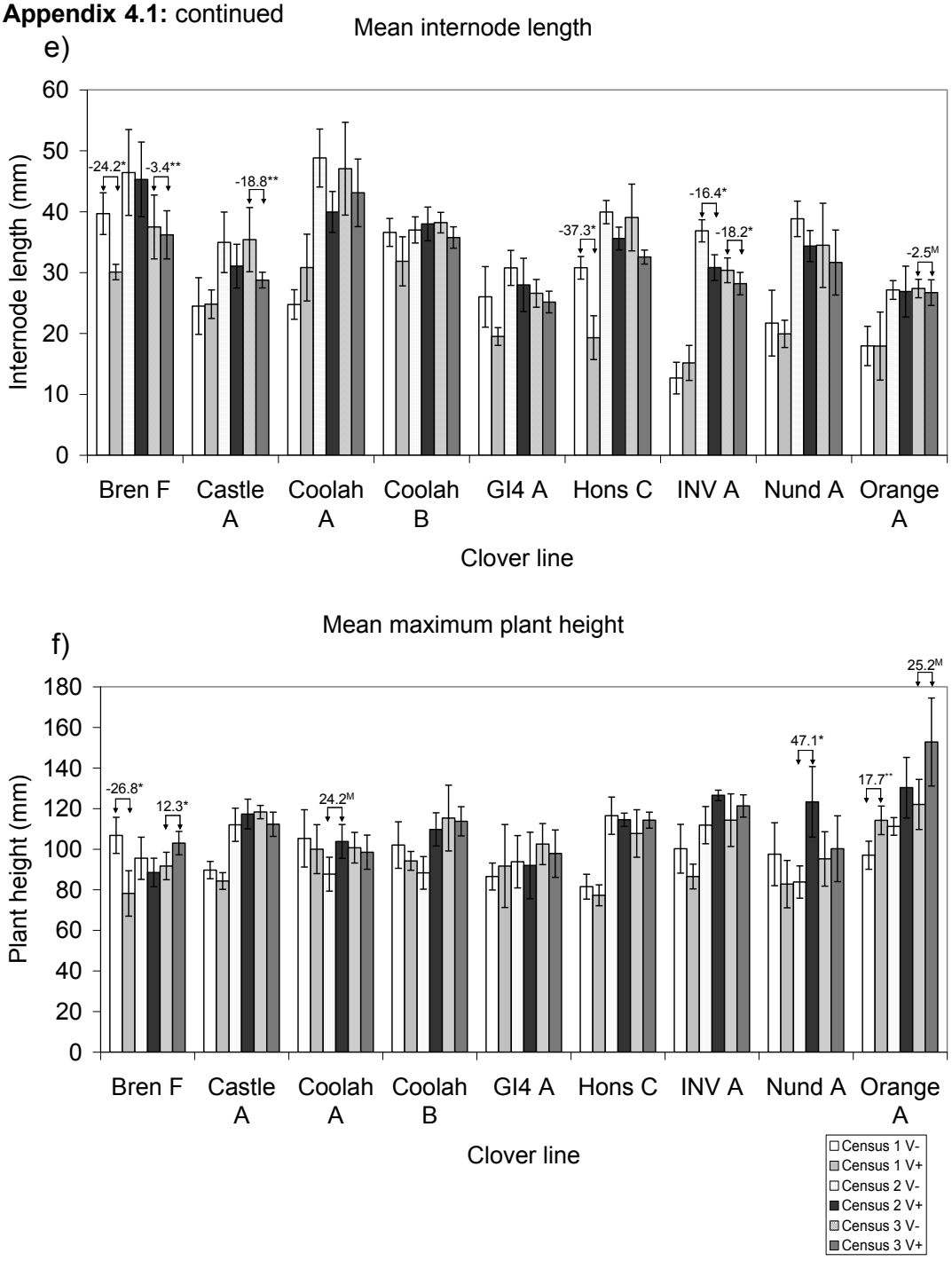

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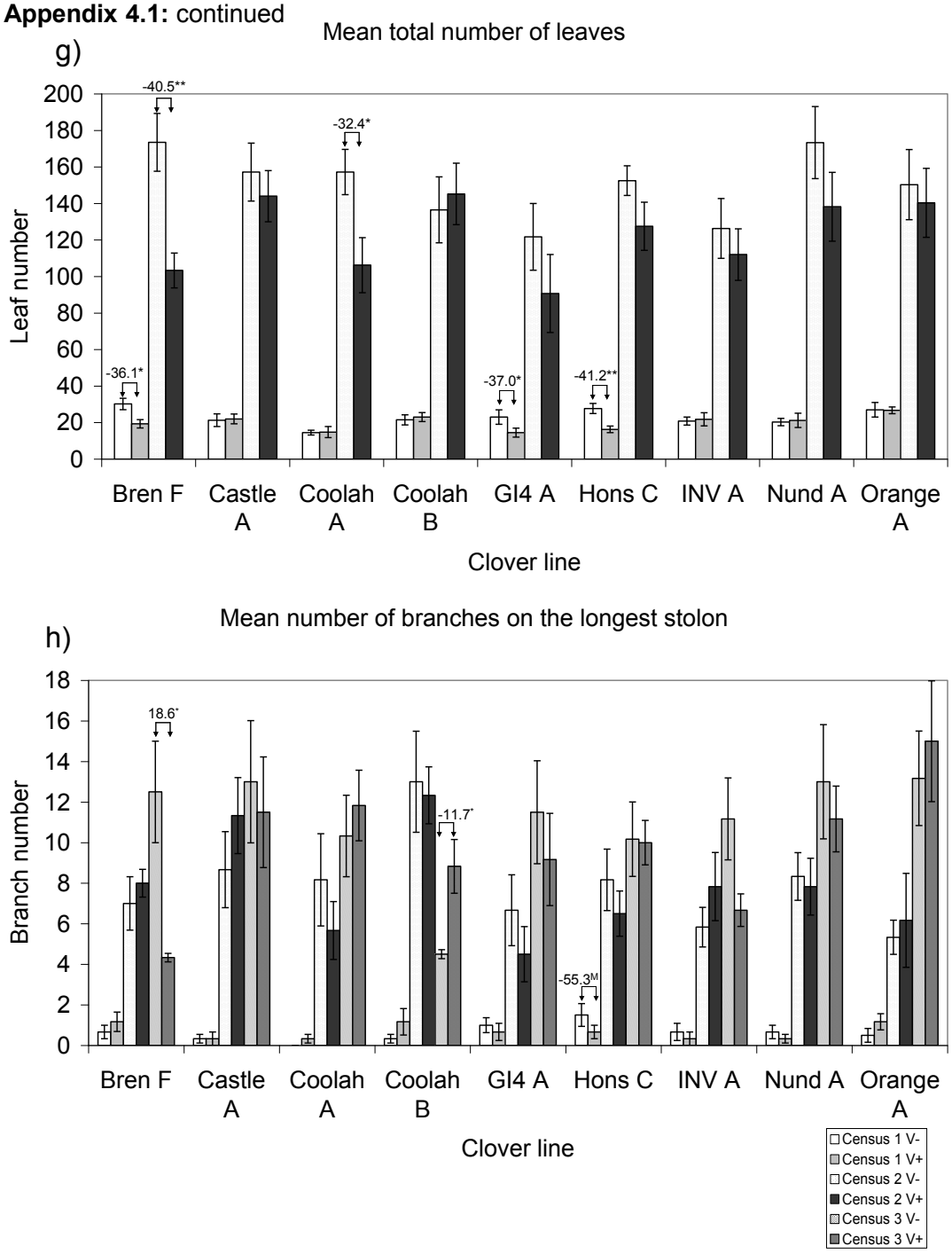
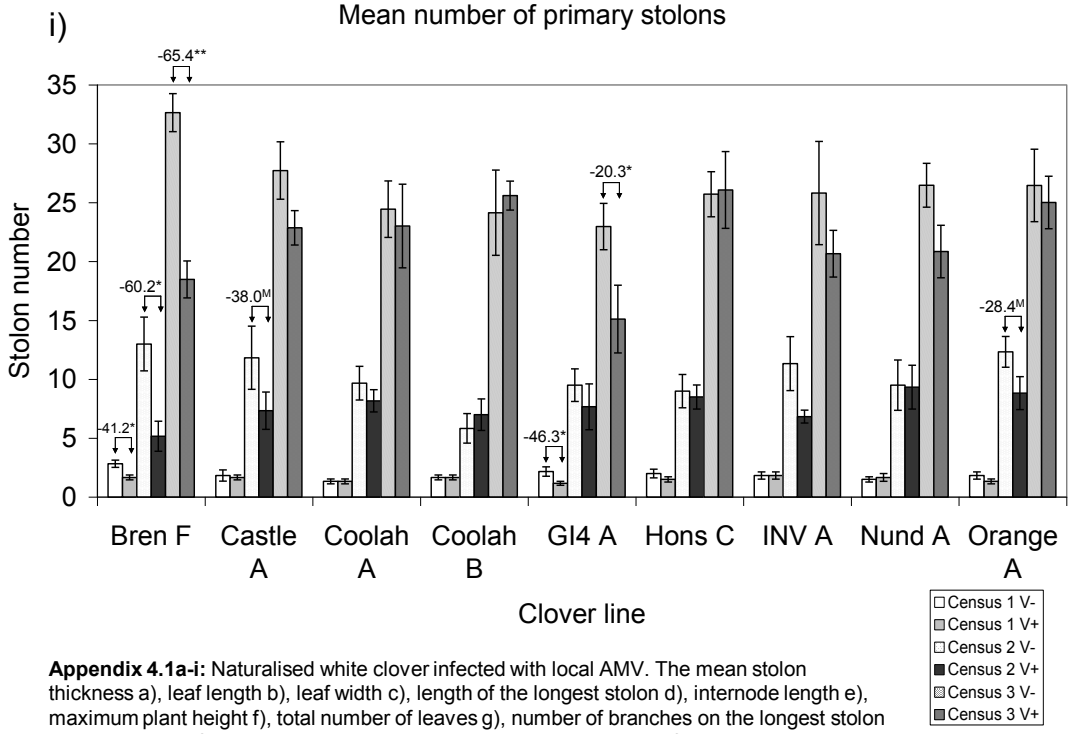


Appendix 4.1: continued



Appendix 4.1a-i: Naturalised white clover infected with local AMV. The mean stolon thickness a), leaf length b), leaf width c), length of the longest stolon d), internode length e), maximum plant height f), total number of leaves g), number of branches on the longest stolon h) and number of primary stolons i) measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth) are presented. The total number of leaves g) was only measured at census 1 and 2. Light coloured bars represent the mean value of virus negative (V-) clover lines at the 3 census dates and grey bars represent the mean value of clover lines infected with local AMV (V+) at the 3 census dates. The standard error of the mean is represented by error bars. The percentage relative virus effect (RVE= ((V+/V-)-1)*100) for each clover line is presented in Appendix 4.2. Significant V-/V+ contrasts are indicated by * (*P* = 0.01-0.05), ** (*P* = 0.001-0.01) or *** *(P* <0.001). Contrasts with marginal *P* values (0.05-0.1) are indicated by M.

Appendix 4.2

a) Relative virus effects of the variables presented in Appendix 4.1a-i. The percentage relative virus effect (RVE=(V+/V-)-1)*100) is based on the means of the virus free plants (V-) and AMV infected plants (V+) for each variable presented in Figure 6. C1, C2, and C3 represent the three census dates: census 1 (after one month of growth), census 2 (two months of growth) and census 3 (three months of growth).

	Mean stolon thickness			Mean leaf length			Mean leaf width			Mean length of the longest stolon			Mean internode length			Mean maximum plant height		
Clover line	C 1	C 2	C 3	C 1	C 2	C 3	C 1	C 2	C 3	C 1	C 2	C 3	C 1	C 2	C 3	C 1	C 2	C 3
Bren F	-9.7 M	13.2 *	13.7	-11.5 M	-2.0	4.7	-5.8	-2.7	1.1	-7.6	-8.1	-4.2	-24.2 *	-2.4	-3.4 **	-26.8 *	-7.3	12.3 *
Castle A	1.7	5.1	11.8	-5.0	-1.0	-5.6	-6.0	-4.6	-8.5	-5.3	-12.7	-22.5 *	1.2	-11.2	-18.8 **	-6.0	4.7	-5.1
Coolah A	-7.8	8.4 M	10.2 M	-3.1	-7.4	-15.7	-9.4	-3.0	-17.6 *	-1.2	-18.8 M	-21.4 M	24.4	-18.2	-8.4	-5.0	18.4	-2.2
Coolah B	-0.1	1.2	3.7	4.3	13.8	9.0	9.0	4.2	10.3	-5.4	-10.1	-8.2	-12.9	2.7	-6.4	-7.6	24.2 M	-1.4
GI4 A	-5.3	-6.3	-11.6 *	0.8	-4.5	-3.3	-6.7	-7.7	-9.5	-29.1 M	-21.5 M	-19.8 M	-25.0	-9.1	-5.4	6.0	-1.9	-4.6
Hons C	-2.6	-1.8	-3.0	-17.9 M	-4.5	-7.2	-23.9 *	1.4	0.6	-29.9 M	-18.9	-15.3	-37.3 *	-10.9	7.2	-5.2	-1.7	6.1
INV A	-10.3 M	9.6 *	14.1	-5.6	-10.9	-6.3	-18.8 M	-11.4	-3.7	52.0	-1.2	21.5	19.6	-16.4 *	-18.2 *	-13.7	13.1	6.1
Nund A	1.0	-17.3	8.2	-7.4	4.6	-3.8	-8.7	2.2	-5.5	-9.6	-7.7	-11.2	-8.2	-11.5	15.6	-15.1	47.1 *	5.3
Orange A	12.9	10.1 *	8.0	34.4 **	-14.0 M	0.5	44.1 **	-9.4 M	-1.4	25.6	1.0	1.7	-0.1	-1.0	-2.5 M	17.7 **	17.2	25.2 M

	Mean total number of leaves			Mean number of branches on the longest stolon			Mean number of primary stolons		
Clover line	C 1	C 2	C 3	C 1	C 2	C 3	C 1	C 2	C 3
Bren F	-36.1 *	-40.5 **	N/A	74.6	14.3	18.6 *	-41.2 *	-60.2 *	-65.4 **
Castle A	3.3	-8.4	N/A	0.0	30.7	2.9	-9.4	-38.0 M	-11.5
Coolah A	2.1	-32.4 *	N/A	100.0	-30.6	-52.3	0.0	-15.5	14.5
Coolah B	7.0	6.4	N/A	254.5	-5.2	-11.7 *	0.0	20.0	96.2
GI4 A	-37.0 *	-25.5	N/A	-33.0	22.6	-51.6	-46.3 *	-19.3	-20.3 *
Hons C	-41.2 **	-16.4	N/A	-55.3 M	-20.4	-16.4	-24.9	-5.6	-1.7
INV A	4.8	-11.3	N/A	-50.7	34.3	42.9	0.0	-39.7	-40.2
Nund A	4.4	-20.3	N/A	-50.7	-6.0	10.9	10.8	-1.7	-14.1
Orange A	-1.1	-6.7	N/A	134.0	15.8	2.9	-27.1	-28.4 M	13.9

M*P* value of V-/V+ contrast is marginal (0.05-0.1)

**P* value of V-/V+ contrast is 0.01-0.05

***P* value of V-/V+ contrast is 0.001-0.01

Appendix 4.3

a) Supplementary growth trial: two naturalised clovers infected with local AMV lines. The mean above ground dry weight (Agdrywt), root dry weight (Rootwt), total dry weight (Totwt) and root to shoot ratio (Rsratio) measured at the final census (after 3 months of growth) were evaluated by analysis of variance. The model R^2 and model effects (clover (C), virus (V) and clover x virus (C x V)) are presented. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are shown with the percentage relative virus effect (RVE=(V+/V-)-1)*100).

		Model effect			Means			
Variable	Model R^2	C	V	C x V	V-	V+	RVE (%)	V+/V- Contrast *P*
Agdrywt	0.55	0.007	0.152	0.664	0.62	0.45	-27.07	0.152
Rootwt	0.52	0.170	0.167	0.101	4.19	3.26	-22.20	0.167
Totwt	0.51	0.111	0.162	0.139	4.81	3.71	-22.87	0.162
Rsratio	0.72	<0.001	0.509	0.077	0.14	0.14	-4.53	0.509

b) Repeated measures analysis of variance of supplementary growth trial: two naturalised clovers infected with local AMV lines. The mean stolon thickness (StThick), length of the longest stolon (StolL), leaf length (Lfleng), leaf width (Lfwid), maximum plant height (Maxht) and internode length (Inleng) measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth) are shown. The mean leaf number (Lvs) was only measured at census 1 and 2, therefore means, RVE and contrasts are not available (N/A) at census 3. The model R^2 and model effects (clover (C), virus (V), time (T), clover x virus (C x V), clover x time (C x T), virus x time (V x T) and clover x virus x time (C x V x T)) are presented. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are shown with the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each census.

									Census 1				Census 2				Census 3			
		Model Effect							Means				Means				Means			
Variable	Model R^2	C	V	T	C x V	C x T	V x T	C x V x T	V-	V+	RVE (%)	V+/V- Contrast *P*	V-	V+	RVE (%)	V+/V- Contrast *P*	V-	V+	RVE (%)	V+/V- Contrast *P*
StThick	0.557	<0.001	0.544	0.065	0.314	0.944	0.608	0.372	1.95	2.04	4.66	0.553	2.00	1.99	-0.35	0.929	1.83	1.84	0.33	0.925
StolL	0.949	0.002	0.009	<0.001	<0.001	0.009	0.062	0.011*	71.30	47.10	-33.94	0.068	244.20	159.30	-34.77	0.004	384.50	296.80	-22.81	0.030
Lfleng	0.734	<0.001	0.054	0.046	0.017	0.244	0.828	0.063	18.34	16.69	-9.00	0.200	16.71	15.35	-8.14	0.131	16.50	15.30	-7.27	0.154
Lfwid	0.649	0.121	0.363	0.816	0.056	0.590	0.700	0.279	15.86	15.09	-4.85	0.553	16.03	15.00	-6.43	0.297	15.85	15.58	-1.70	0.734
Lvs	0.876	0.604	0.318	<0.001	0.129	0.237	0.370	0.099	10.70	9.30	-13.08	0.390	50.20	42.20	-15.94	0.347	N/A	N/A	N/A	N/A
Maxht	0.682	0.002	0.509	0.774	0.435	0.413	0.710	0.065	90.80	90.80	0.00	0.993	94.10	90.10	-4.25	0.595	98.00	90.20	-7.96	0.320
Inleng	0.815	<0.001	0.046	<0.001	0.024	0.750	0.132	0.220	16.88	11.93	-29.32	0.163	29.33	22.63	-22.84	0.030	26.38	26.57	0.72	0.921

*A significant difference (<0.05) in the clover x virus x time interaction between clovers Stoney D and Bren F was observed between census 1 and 2 for StolL.

Appendix 4.3: continued

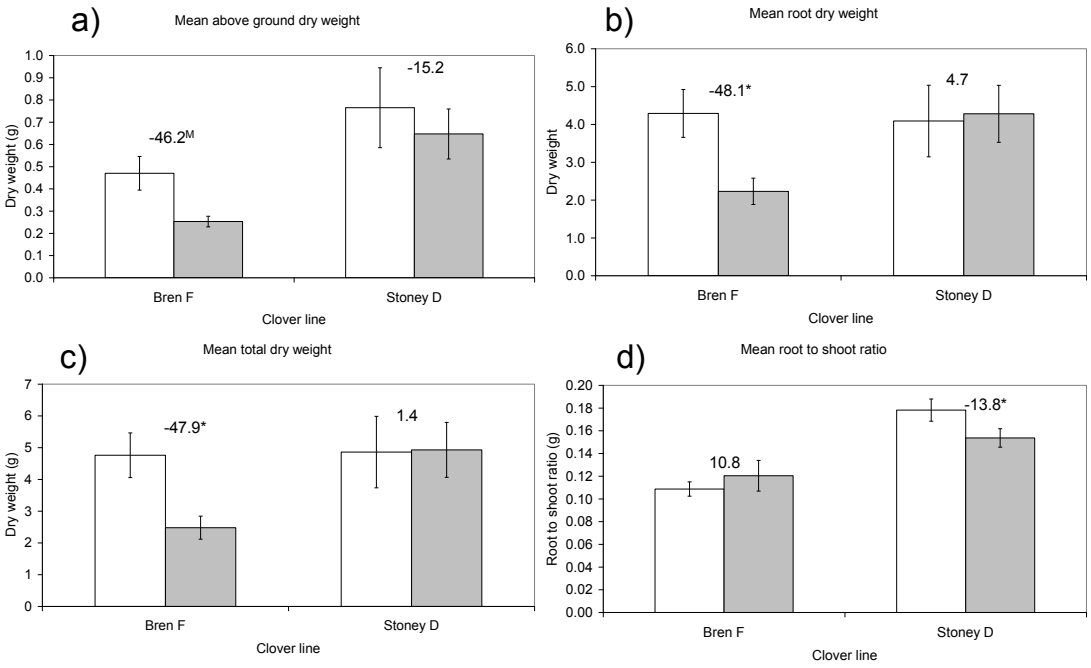
c) General linear mixed model analysis (Poisson distribution, logarithm link function) of supplementary growth trial: two naturalised clovers infected with local AMV lines. All variables were measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth). The mean total inflorescence number (FHTot), number of branches on the longest stolon (BranchNo) and number of primary stolons (PriStNo) at each census are shown. The model effects (clover (C), virus (V), clover x virus (C x V)) are presented. The means of the virus free plants (V-) and AMV infected plants (V+) for each variable are shown with the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each census. RVE cannot be calculated for FHTot at census 3 and PriStNo at census 1 because V-= 0. Data was not available (N\A) to calculate model effects and contrasts for BranchNo at census 1, and FHTot at census 3.

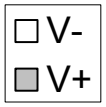
	Census 1							Census 2							Census 3						
	Model Effect			Means			V+/V- Contrast	Model Effect			Means			V+/V- Contrast	Model Effect			Means			
Variable	C	V	C x V	V-	V+	RVE (%)	*P*	C	V	C x V	V-	V+	RVE (%)	*P*	C	V	C x V	V-	V+	RVE (%)	*P*
FHTot	0.023	0.089	1.000	0.08	0.25	200.12	0.544	<0.001	<0.001	<0.001	0.08	0.00	-100.00	0.333	N/A	N/A	N/A	0.00	0.00	N/A	N/A
BranchNo	N/A	N/A	N/A	0.00	0.00	N/A	N/A	0.206	0.773	0.138	5.67	5.17	-8.82	0.754	0.351	0.558	0.066	6.17	7.08	14.85	0.539
PriStNo	1.000	1.000	0.064	1.00	1.00	0.00	1.000	0.252	0.565	0.452	4.25	3.50	-17.65	0.593	0.314	0.971	0.327	5.17	5.25	1.61	0.966

d) Variables analysed using repeated measures analysis of variance (Appendix 4.3b) that had significant model effects of interest from the supplementary growth trial: Experiment 2, 2 naturalised clovers infected with local AMV lines were re-analysed by analysis of variance at separate census dates. The model R^2 and model effects (clover (C), virus (V), clover x virus (C x V)) are presented for the mean internode length (lnleng), leaf length (Lfleng) and stolon length (StolL) measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth). The means of the virus free plants (V-) and AMV infected plants (V+) and the percentage relative virus effect (RVE=(V+/V-)-1)*100) for each variable are presented in Appendix 4.3b.

	Census 1				Census 2				Census 3			
		Model Effect				Model Effect				Model Effect		
Variable	Model R^2	C	V	C x V	Model R^2	C	V	C x V	Model R^2	C	V	C x V
lnleng	0.563	0.025	0.163	0.097	0.641	0.003	0.030	0.019	0.718	<0.001	0.921	0.488
Lfleng	0.509	0.145	0.200	0.009	0.579	0.009	0.131	0.241	0.727	<0.001	0.154	0.445
StolL	0.602	0.019	0.068	0.015	0.761	0.005	0.004	<0.001	0.703	0.002	0.030	0.003

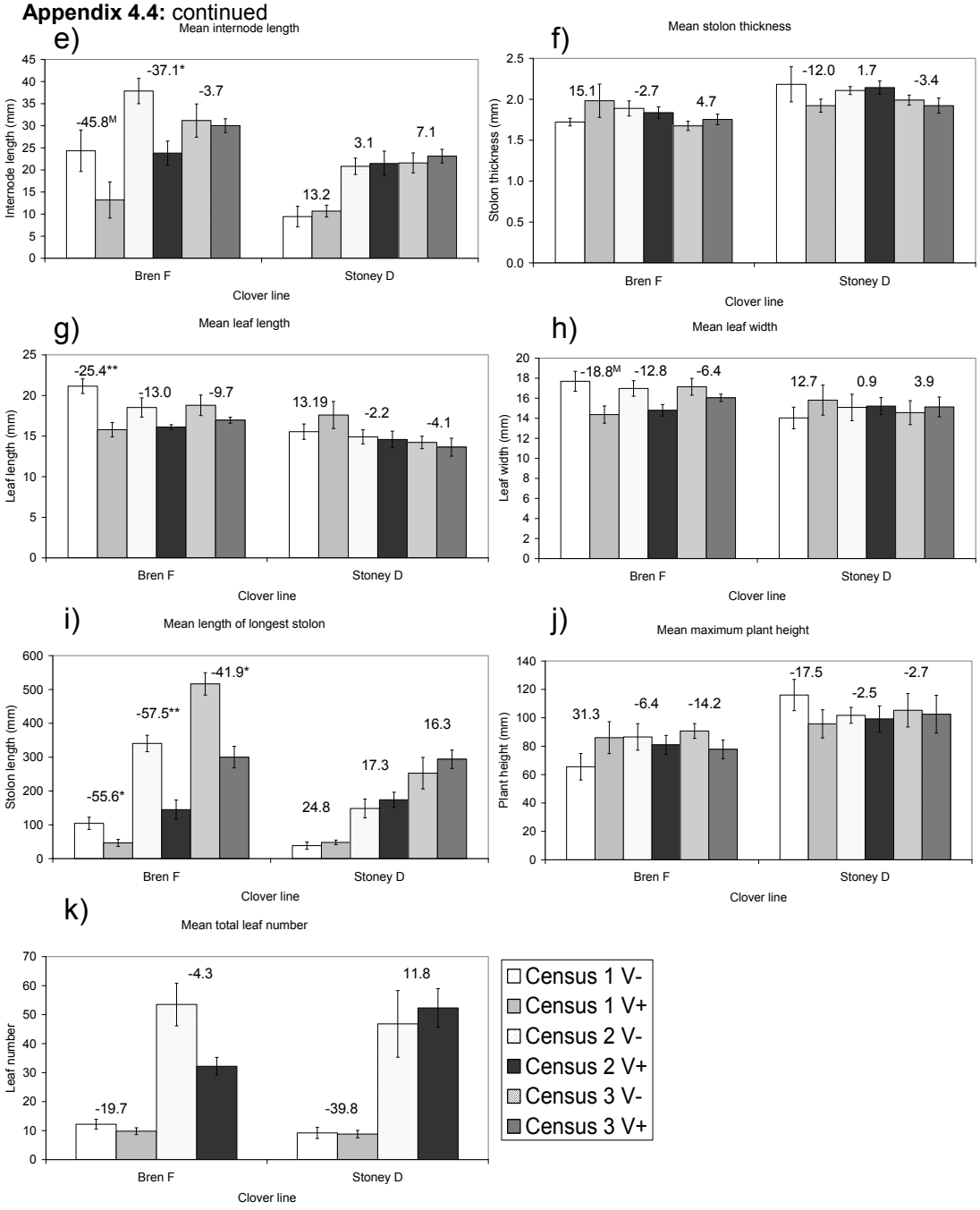
Appendix 4.4



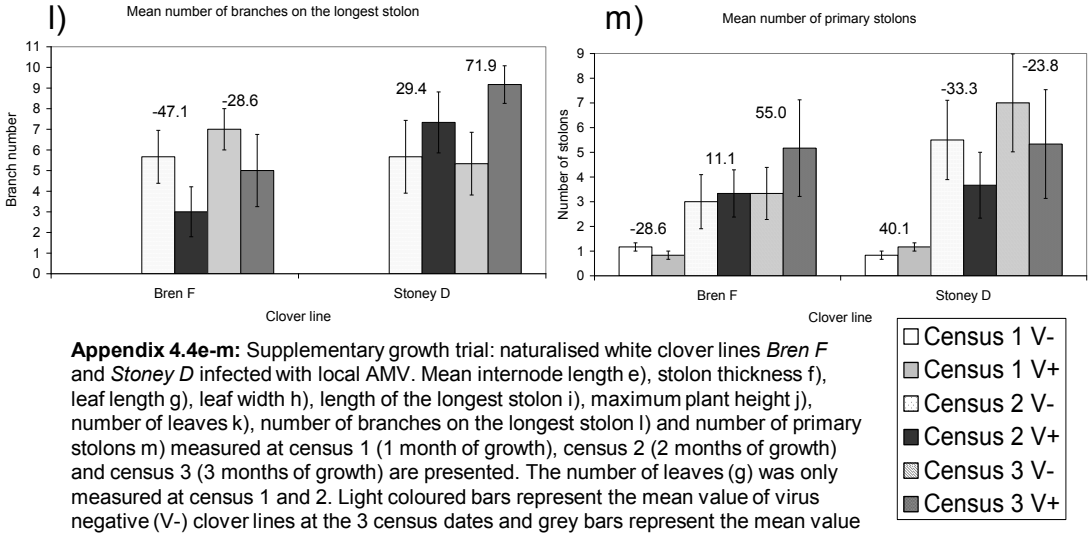


Appendix 4.4a-d: Supplementary growth trial: naturalised white clover lines *Bren F* and *Stoney D* infected with local AMV. Mean above ground dry weight a), dry root weight b), total dry weight c) and root to shoot ratio d) measured at the final census date (after 3 months growth) are presented. White bars represent the mean value of virus negative (V-) clover lines, grey bars represent the mean value of clover lines infected with local AMV (V+) and the standard error of the mean is represented by error bars. The percentage relative virus effect (RVE= ((V+/V-)-1)*100) for each clover line is the value presented above V- and V+ pairs of bars. Significant V-/V+ contrasts are indicated by * (*P* = 0.01-0.05) and contrasts with marginal *P* values (0.05-0.1) are indicated by M.

Appendix 4.4: continued



Appendix 4.4: continued



Appendix 4.4e-m: Supplementary growth trial: naturalised white clover lines *Bren F* and *Stoney D* infected with local AMV. Mean internode length e), stolon thickness f), leaf length g), leaf width h), length of the longest stolon i), maximum plant height j), number of leaves k), number of branches on the longest stolon l) and number of primary stolons m) measured at census 1 (1 month of growth), census 2 (2 months of growth) and census 3 (3 months of growth) are presented. The number of leaves (g) was only measured at census 1 and 2. Light coloured bars represent the mean value of virus negative (V-) clover lines at the 3 census dates and grey bars represent the mean value of clover lines infected with local AMV (V+) at the 3 census dates. The standard error of the mean is represented by error bars. The percentage relative virus effect (RVE= ((V+/V-)-1)*100) for each clover line is the value presented above V- and V+ pairs of bars. Significant V-/V+ contrasts are indicated by * (*P* = 0.01-0.05), ** (*P* = 0.001-0.01) and contrasts with marginal *P* values (0.05-0.1) are indicated by M.

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